Elucidation of cellular mechanisms that may contribute to polyphenol-induced effects on neutrophil chemokinesis

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DECLARATION

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March 2017

Abstract

Grape-derived products are high in polyphenols and are known to have anti-oxidant and anti-inflammatory effects. *In vivo* studies in the context of muscle injury-induced inflammation have proven grape seed-derived proanthocyanidin oligomers (PCO) to benefit recovery by modulation of neutrophil infiltration into damaged tissue, thereby reducing secondary damage. Also, in these models, PCO have been shown to facilitate an early anti-inflammatory macrophage phenotype shift, which resulted in faster resolution of inflammation and shortened recovery time. However, these results have not been investigated in a human model and the specific molecular targets of PCO is not clear.

This study therefore aimed to investigate potential molecular targets of PCO in a normally healthy population. In addition, given anecdotal concern about consumer safety, a secondary aim was to investigate haematological effects of short-term PCO supplementation in humans. A limited haematology assessment was included to address these concerns.

Eighteen normally healthy volunteers between the ages of 18-25 years old (13 female and 5 male) were subjected to daily oral supplementation with 140mg of PCO for a 2-week period. Blood samples were taken at baseline (day 0), as well as on days 7 and 14. Day 0 and 14 samples were comprehensively analysed for *in vitro* neutrophil chemokinetic capacity towards a chemotaxin (fMLP) using live cell tracking software, as well as neutrophil expression levels of adhesion molecules (ICAM-1, VCAM-1 and CD66b) by flow cytometry and cell polarisation factors (ROCK, PI3K) using immunohistochemistry. Macrophage expression of markers indicative of different phenotypes were assessed using flow cytometry. In addition, day 7 samples were assessed for PCO-induced effects on general haematology and hemostasis.

No adverse effects of PCO was evident. A novel neutrophil migration assay was developed to allow immunohistochemistry staining on chemokinetic neutrophils, allowing assessment of molecular role players involved in chemokinesis and actual movement capacity on the same cells. Although PCO supplementation had no evident effect on neutrophil chemokinesis or adhesion molecule expression, the increase in ROCK co-localisation with PI3K under stimulated conditions was prevented, while ROCK expression itself tended to be decreased. Macrophage phenotype markers CD274 and MPO – both indicative of a pro-inflammatory M1 phenotype – was normalised after PCO treatment.

We conclude that the PCO product employed in the current study, was safe for consumption. Furthermore, data indicates that neutrophil chemokinetic capacity may be optimised by PCO via modulation of the ROCK-PI3K-PTEN system, which results in better front-rear synchronisation of cell polarisation and thus movement. Finally, data confirms earlier reports in rodents, of a direct effect on macrophages to achieve a relatively anti-inflammatory phenotype predominance.

Uittreksel

Produkte afgelei van druiwe bevat hoë konsentrasies polifenole en is bekend vir hul anti-oksidant- en anti-inflammatoriese effekte. In vivo-studies oor spierbeskadiging en verwante inflammasie, het bewys dat druif-verwante polifenole (PCO) herstel stimuleer deurdat dit infiltrasie van neutrofiele na die beseerde weefsel beperk, wat sekondêre skade verminder. Hierdie modelle het ook gewys dat PCO ‘n vroëë anti-inflamatoriese fenotipeverskuiwing in makrofage bewerkstelling, wat lei tot ‘n vinniger afname in inflammasie en ‘n verkorte hersteltyd. Hierdie effekte is egter nog nie voldoende in mensmodelle nagevors nie en die spesifieke molekulêre teikens is onbekend.

Hierdie studie het gepoog om die teikens van PCO in ‘n gesonde populasie te ondersoek. Gegewe berigte van gebruikerskommer rakende veiligheid, was ‘n verdere doel om die effek van korttermyn supplementasie met PCO op die algemene hemotologie and hemostase te ondersoek.

Agtien gesonde vrywilligers, tussen die ouderdomme van 18-25 jaar (13 vroulik en 5 manlik), is onderwerp aan ‘n daaglikse mondelinge aanvulling van 140mg PCO oor ‘n tydperk van twee weke. Bloedmonsters is geneem voor supplementasie (dag 0), asook na 7 en 14 dae van aanvullling. Dag 0 en 14 monsters is deeglik ondersoek vir in vitro neutrofiel chemokinetiese bewegingskapasiteit in die rigting van ‘n chemotaksin (fMLP) deur van lewendesel-mikroskopie asook neutrofiel uitdrukkingsvlakke van adhesiemolekule (ICAM-1, VCAM-1 en CD66b), deur vloeisitometrie en selpolarisasiefaktore (ROCK, PI3K) met immunohistochemie gebruik te maak. Makrofaaguitdrukking van merkers wat verskilledne fenotipes aandui is met vloeisitometrie bepaal. Dag 7 monsters is ontleed vir PCO-verwante effekte op algemene hemotologie en hemostatse-aanwysers.

Geen newe-effekte van PCO inname was waarneembaar nie. ‘n Nuwe neutrofielmigrasietoets is ontwikkel om immunohistochemiese ondersoeke op migrerende neutrofiele te kon uitvoer, om die bewegingskapasiteit en molekulêre rolspelers in dieselfde selle te assesseer. Alhoewel PCO aanvullings geen sigbare effek op neutrofielbeweging of die uitdrukking van adhesiemolekule gehad het nie, was ROCK-uitdrukking op neutrofiele laer, terwyl die verhoodge gesamentlike uitdrukking van ROCK met PI3K, wat gepaard gaan met stimulasie, betekenisvol ge-inhibeer is. Die makrofaag merkers CD274 en MPO, beide aanwysers van die pro-inflammatoriese M1 fenotipe, het na PCO aanvulling genormaliseer.

Die gevolgtrekking is dat die PCO-produk veilig is vir gebruikers. Verder dui data aan dat neutrofiel-chemotaktiese kapasiteit ge-optimaliseer word deur PCO, deurdat dit die ROCK-PI3K-PTEN sisteem moduleer, om sodoende beter voorpunt-agterpuntsinchronisasie van selpolarisasie en dus beweging, teweeg te bring. Die data verkry in hierdie studie onderskraag vroëëre eksperimentele dat verkry in knaagdiere, van ‘n direkte effect van PCO op makrofage sodat die anti-inflammatoriese fenotipe oorheers.

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List of Abbreviations

ADP	Adenosine diphosphate
aPTT	Activated clotting time
ATP	Adenosine triphosphate
BBB	Blood Brain Barrier
CHD	Coronary heart disease
C5a	Complement Component
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EGCG	Epigallocatechin 3-gallate
FBS	Fetal Bovine Serum
fMLP	N-Formylmethionine-leucyl-phenylalanine
GAP	GTPase-activating Protein
GEF	Guanine Nucleotide-exchange Factor
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor
GSPE	Grape seed proanthocyanidin extract
GTP	Guanosine Triphosphate
GTPase	GTP Phosphatase
HLA-DR	Human Leukocyte Antigen – Antigen D Related
HMGB1	High mobility group box 1
HREC	Health Research Ethics Committee
HUVEC	Human Umbilical Vein Endothelial Cell
ICAM-1	Intercellular Adhesion Molecule 1
INF-γ	Interferon gamma

IL-1β	Interleukin-1β
IL-3	Interleukin-3
IL-4	Intereukin-4
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-10	Interleukin 10
INR	International Normalized Ratio
JAM-A	Junctional Adhesion Molecule A
LPS	Lipopolysaccharides
LTB4	Leukotriene B4
MAPK	Mitogen-activated protein kinase
MCH	Mean Cell Haemoglobin
MCHC	Mean Corpuscular Haemoglobin Concentration
MCV	Mean Corpuscular Volume
mDia1	Diaphanous-related Formin-**1**
MFI	Median Fluorescent Intensity
MIP	Macrophage inflammatory protein
MPO	Myeloperoxidase
MR	Mannose receptor
M1	Classically Activated M1 Phenotype Macrophage
M2	Alternatively Activated M2 Phenotype Macrophage
NF-κB	Nuclear factor kappa-light-chain enhancer of activated B cells
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCO	Proanthocyanidolic oligomers

PI	Phosphatidylinositol
PI3K	Phosphatidylinositol 3-kinase
PI3P	Phosphatidylinositol 3-phosphate
PI(3,4)P2	Phosphatidylinositol 3,4-bisphosphate
PI(3,4,5)P3	Phosphatidylinositol 3,4,5-triphosphate
PI(3,5)P2	Phosphatidylinositol 3,5-bisphosphate
PI4P	Phosphatidylinositol 4-phosphate
PIP3	Phosphatidylinositol 3,4,5-triphosphate
pMLC	Phosphorylated myosin light chain
PT	Prothrombin time
PTEN	Phosphatidylinositol 3-phosphotase
RDW	Red cell Distribution Width
ROCK	Rho-associated coil-containing protein kinase
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute medium
SHIP1	SH2 domain-containing inositol 5-phosphatase
SEM	tandard Error of the Mean
SR	Scavenger receptor
SS	Systemic sclerosis
TGF-ß	Transforming Growth Factor beta
TLR	Toll-like Receptor
TNF-α	Tumour Necrosis Factor α
VCAM-1	Vascular Cell Adhesion Molecule 1

Units of Measure

%	percentage
°C	degrees Celsius
µg	microgram
µg/cm^2	microgram per square centi
µg/ml	microgram per millilitre
µl	microliter
µm	micrometre
µM	micromolar
fl	femtoliter
g	gravitational acceleration
g/dL	Grams per decilitre
L/L	Liter/Liter
g/L	gram per Liter
g/mol	gram per mole
h	hour
L	Liter
M	molar
MFI	Median Fluorescent Intensity
Mg	milligram
mg/ml	milligram per millilitre
min	minutes
ml	millilitre
mm	millimetre

mM	millimolar
ng/ml	nanogram per millilitre
nm	nanometre
nM	nanomolar
pg	picogram
kDa	kilo Dalton
RPM	revolutions per minute
Sec	seconds

1. CHAPTER 1: INTRODUCTION

Due to advances in medicine in general, the world population are increasing in age as average lifespan is increasing (Naghavi *et al.*, 2015). Advanced age is characterised by a relatively more pro-inflammatory profile and increased oxidative stress. Furthermore, modern society is increasingly burdened by inflammation-related illnesses. Inflammation is now a commonly accepted aetiological factor in lifestyle diseases such as cardiovascular disease, type II diabetes and depression, as well as in cancer. In terms of the inflammatory and oxidative stress profiles of these diseases, they can all be classified as manifestations of "accelerated ageing". It is thus imperative to find solutions to the increasing burden on the health sector in this context.

Various therapeutic targets have been researched which may assist in the elevation of chronic inflammatory diseases. There are many non-steroidal as well as natural products that assist in the clearance of secondary damage caused by inflammation. New products are constantly being pushed onto the market without being thoroughly tested and scientists are currently under immense pressure to try and keep up. Also, natural products are becoming an extremely popular choice in mediating chronic inflammatory diseases. Natural products such as polyphenols have showen potential in both an anti-inflammatory as well as anti-oxidative capacity. The issue however, is that natural products are not as thoroughly researched as pharmaceutical products and this could be compromising to a person's health if products are not properly understood.

In order to evaluate the potential of experimental treatment or supplements on these systems, it is necessary to understand the processes and mechanisms at play. For example, inflammation and oxidative stress are two intricately interlinked processes (Petersen *et al.*, 2016). Although oxidative stress is not a topic of this thesis, it is important to note that inflammation – and particularly the neutrophil phase – is known to result in oxidative stress-related secondary damage to healthy tissue, via oxygen radical release by neutrophils during oxidative burst (Tidball, 2005). Also, particularly in aged individuals, directional inaccuracy of movement of neutrophils toward a chemotactic signal (also termed chemokinesis), have been implicated in further tissue damage (Sapey *et al.*, 2014).

Conversely, anti-oxidant treatment has been associated with a more desirable inflammatory profile in various models (Petersen *et al.*, 2016). In addition, our group have recently shown anti-oxidant treatment to improve neutrophil directional accuracy (Petersen and Smith, 2016). Briefly, human donor blood was treated with a grape seed-derived polyphenol *in vitro*, before assessments were made. Thus, the purpose of this thesis was to expand on these results by firstly performing an *in vivo* supplementation study and secondly to include investigation of parameters which may influence directional accuracy of neutrophil chemokinesis. This includes brief investigation of potential contributions by macrophages, which is known to secrete a number of chemokines and cytokines affecting the inflammatory process (Smith *et al.*, 2008), as they are known to co-exist with neutrophils as major role players during inflammation.

In the next chapter, and overview of the phagocytic immune cells and mechanisms at play during chemokinesis, will be discussed. In addition, an overview of the literature related to plant-derived products in this context, will be provided.

2. CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

The immune response, arguably most important to both innate and adaptive immunity, is inflammation. The main purpose of inflammation is to send phagocytes to sites of infection or injury. At the sites of infection, phagocytes may isolate, destroy or disable the invaders; remove debris; and prepare the healing and repair process (Sherwood, 2015). The inflammatory process follows a similar trend regardless of the trigger (bacterial, chemical injury or mechanical trauma). The acute inflammatory response lasts for a short duration and is characterised by the accumulation of leukocytes, cytokines, neutrophil infiltration and fluid exudation (Sherwood *et al.*, 2004). When this initial inflammatory response persists for longer periods, it results in chronic inflammation (either low grade or severe) states. Chronic low grade inflammation leads to cumulative oxidative stress and secondary damage to healthy tissue and has been implicated in various modern diseases of lifestyle (Sherwood, 2015).

The process of inflammation is initiated through the first event of vasodilation, which leads to a pyrogenic reaction and results in the site of injury becoming red and warm. The small blood vessels with thin vascular endothelium normally permit free exchange of water and small molecules between blood and tissue spaces; but limit the passage of plasma proteins. However, after injury, the permeability of the injured area increases and consequently allows plasma proteins, leukocytes and more fluid from the blood to secrete into the tissue spaces (Sherwood *et al.*, 2004) .Leukocytes, especially neutrophils and monocytes, migrate out of the blood vessels and accumulate in vast numbers at the site of injury. Neutrophils predominate the first 6 to 24 hours in acute inflammation and are later replaced by monocyte migration within 24 - 48 hours. However, the patterns of leukocytes exudates differ depending on various factors of initiation (viral or bacterial infection or hypersensitivity (Sherwood *et al.*, 2004). Resident macrophages are converted monocytes that may exhibit two distinct phenotypes. The M1/M2 phenotypes describe the two major and opposing behaviours of macrophages. The M1 phenotype inhibits cell proliferation and causes tissue damage while the M2 phenotype promotes cell proliferation and tissue repair (Mills, 2012). Cellular debris from the damaged tissue is removed by the infiltrating neutrophils before

satellite cells proliferate to replace the previously damaged and phagocytized tissue. In addition to phagocytosis, neutrophil invasion and activation can also lead to the release of reactive oxygen species (ROS) and proteases which potentially cause further injury to the affected site (Toumi *et al.*, 2003). **Figure 2.1** illustrates the proposed mechanism of the relation between inflammatory response and the further tissue damage caused by neutrophils.

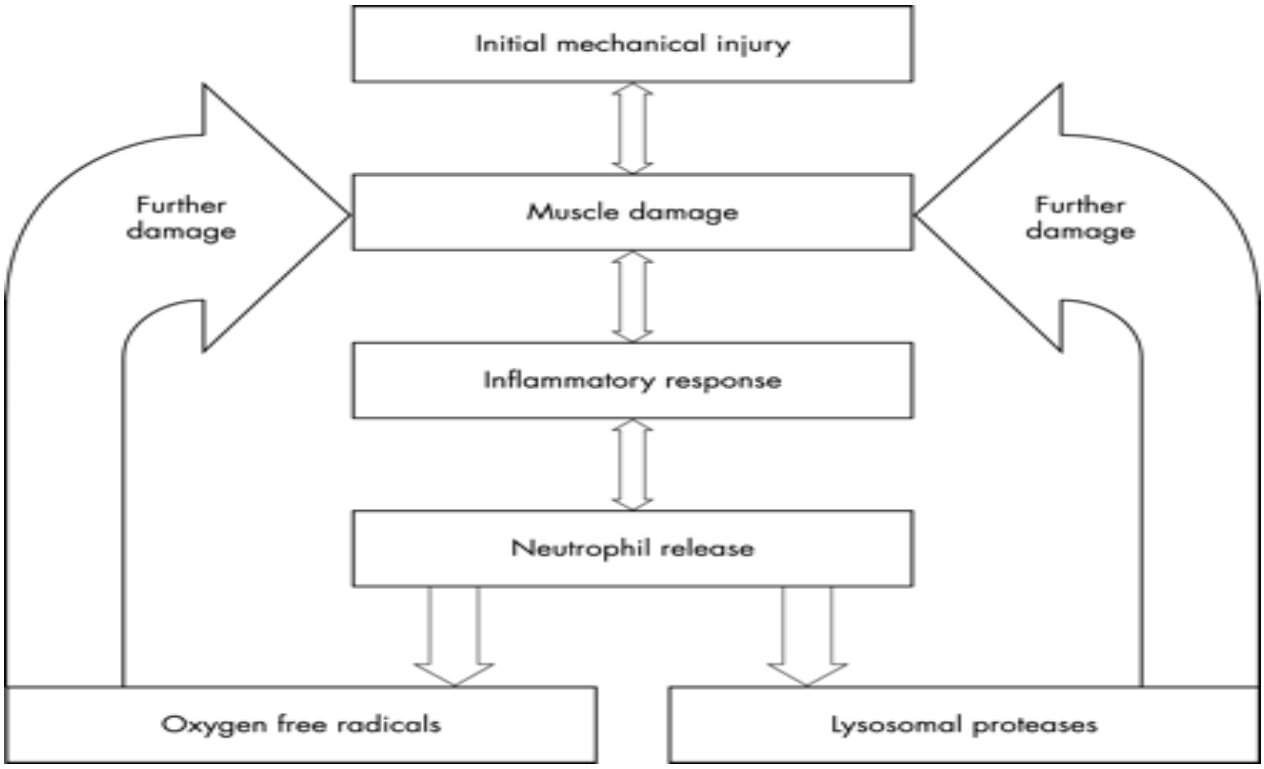


Figure 2.1: Proposed mechanism of the relation between the inflammatory response and further tissue damage. *Neutrophils may promote further damage through the release of oxygen free radicals and lysosomal proteases and elastase* (Toumi *et al.*, 2003).

2.2 Neutrophils

2.2.1 Role in inflammation

Neutrophils protect the body against any type of infection whether bacteria or fungal, and are the body's first line of defence. Once activated, they move swiftly to the site of infection or inflammation and therefore have been identified as key players during the immune response (Smith *et al.*, 2008).

Phagocytosis is the mechanism used by neutrophils (and other phagocytes) to ingest foreign invaders and/or cellular debris at the site of infection/injury (Gambardella *et al.*, 2013). During the process of phagocytosis, neutrophils generate ROS that is involved in degranulation and subsequent release of proteases and inflammatory mediators. Although this process is a necessary step that helps to clear the site from pathogens and cellular debris, it may also results in secondary damage to previously healthy tissue. In fact, up to 80% of total tissue damage after infection/injury can be ascribed to neutrophil secretory products (Tidball, 2005). Furthermore, unused or excess neutrophils undergo apoptosis and have to be removed by pro-inflammatory macrophages *via* phagocytosis. Only when this step has been completed, can resolution of inflammation commence, *via* M2 macrophage action (Järvinen *et al.*, 2005).

Given the potential for neutrophils to exacerbate damage, and the commonly known phenomenon of maximal response of the immune system, it may be possible to limit the capacity of neutrophils to cause secondary damage, without compromising the effectiveness of the response. Specifically, the neutrophil response can be "optimised" in terms of its efficacy so that fewer cells would be required for any given stimulus. One way in which this may be achieved, is *via* the optimisation of neutrophil migration through tissue in terms of speed and/or directional accuracy. In the next sections, I will provide an overview of the mechanisms used for migration, before discussing potential intervention in this context.

2.2.2 Mechanisms of neutrophil migration

Neutrophils are produced in the bone marrow and do not have a particularly long lifespan (<1 day), but when needed they can move out of the bone marrow quickly and in great numbers (Smith *et al.*, 2008), (Gambardella *et al.*, 2013);. Mature neutrophils that are found in the bloodstream can form connections with the blood vessel wall. They do so by interacting with 2 different classes of receptors as well as integrins and selectins (Gambardella *et al.*, 2013), (Hillgruber *et al.*, 2015). The process of extravasation of neutrophils entails slowing down, rolling along the blood vessel wall, attaching to endothelial cells and then transendothelial migration into tissue, which is directed by chemotactic signals released from the site of infection or damage (Smith *et al.*, 2008), (Gambardella *et al.*, 2013), (Hillgruber *et al.*, 2015). For neutrophils to become adherent upon activation, their integrins (mostly β2) need to bind to the proteins within the extracellular matrix (ECM) which in turn connects them to the actin cytoskeleton (Gambardella *et al.*, 2013). β2 integrin-deficient neutrophils do not migrate towards the chemoattractant and therefore cannot form an attachment on the ECM (Gambardella *et al.*, 2013).

2.2.3 Neutrophil adhesion molecules

The movement of immune cells from vascular endothelium into the intravascular space occurs *via* cellular adhesion molecules that guide the process of recruitment, adhesion and translocation of these immune cells (Smith *et al.*, 2008). To ensure increased leukocyte numbers from vascular sites to the site of inflammation, the endothelium needs to be activated by pro-inflammatory cytokines, namely tumor necrosis factor-alpha (TNF-α) or interferon-gamma (INF-γ) (Smith *et al.*, 2008), (Kuntz *et al.*, 2014). Various cellular adhesion molecules are also expressed over different periods to facilitate this process. Abnormalities in the regulation of some of these molecules have been linked to various pathophysiological conditions, such as chronic inflammation, cancer and atherosclerosis (Kuntz *et al.*, 2014). For example, relevant to our topic of migration, cellular adhesion molecule mal-adaptation was shown to contribute to endothelial irregularities in atherosclerosis, an outcome which will no doubt also affect leukocyte migration across endothelial cells (Kusters *et al.*, 2012).

E-selectin is also an adhesion marker, which is responsible for the initial contact between leukocytes and the vascular endothelium. Specifically, E-selectin allows for the first attachment of leukocytes to the vascular endothelium and this is referred to as tethering (Kuntz *et al.*, 2014). The two other adhesion molecules of interest to the present review are intracellular adhesion molecule 1 (ICAM-1) and vascular adhesion molecule 1 (VCAM-1) and are known to be responsible for the strong adhesion and transmigration of immune cells into the intravascular space (Kuntz *et al.*, 2014). ICAM-1 and VCAM-1 are not only expressed on cell surfaces, but also in plasma in a soluble form (Smith *et al.*, 2008). CD66B is one of four granulocyte specific activation antigens that form part of the Cd66 family (Jensen *et al.*, 2012), (Schmidt *et al.*, 2012), (Zhou *et al.*, 2012). Upon stimulation, CD66B expression on neutrophils becomes upregulated and is known to play a pivotal role in adhesion and activation of these cells (Jensen *et al.*, 2012), (Schmidt *et al.*, 2012), (Zhou *et al.*, 2012). A study by Kuntz *et al.*, (2014) involving the use of two types of curly kale preparations on human umbilical vein endothelial cells (HUVECs) reported a reduction of both ICAM-1 (74.6 ± 10.2% and 76.6 ± 7.9%) and VCAM-1 (35.0 ± 14% and 81.66 ± 7.9%) mRNA levels in HUVECs stimulated by both types of curly kale preparations. They concluded that curly kale extracts play a particular role in the modulation of TNF-α stimulated neutrophil adhesion and resulted in the reduction of adherent neutrophils (Kuntz *et al.*, 2014).

It is vital that neutrophils are motile and able to assist in the inflammatory process. Atherosclerosis-associated inflammation was reported to be mediated by a rise in the concentration of soluble adhesion molecules (Roberts *et al.*, 2006); (Petridou *et al.*, 2007). Interestingly, people suffering from chronic diseases such as diabetes, and have taken up regular exercise as a form of therapy were reported to show a decrease in soluble ICAM-1 level, which may contribute to the normalisation of serum lipid and insulin levels as well as a reduction in oxidative stress level observed in the subjects (Roberts *et al.*, 2006). This result indicates how intervention in this context may have significant clinical effectiveness.

Gorina *et al.*, (2014) determined how selectin-independent neutrophil interact with the blood brain barrier (BBB) and focused on the role of β2 integrins and their endothelial ligands ICAM-1, ICAM-2 and junctional adhesion molecule-A (JAM-A). Neutrophil crawling was only seen to be inhibited in the absence of the combined ICAM-1 and ICAM-2 or when all β2 integrins were absent (Gorina *et al.*, 2014). VCAM1 played no role in the interaction between neutrophil and the inflamed BBB (Gorina *et al.*, 2014). Although this data was generated in a

model specific for the BBB, it highlights the importance of assessing multiple parameters and their interactions, rather than just one parameter in isolation.

2.2.4 Chemotaxis

Chemotaxis is made up of cell migration and gradient sensing. The way in which neutrophils accomplish their main goal by moving through various environments is by making use of chemical gradients originating from the target area (Irimia, 2010). Neutrophil chemotaxis is the process by which these cells sense a chemoattractant gradient, then migrate towards the signal (Gambardella *et al.*, 2013). Interestingly, as soon as neutrophils are in the range of an infection, they "choose to" ignore intermediary chemokines even though they make use of 7-transmembrane-spanning G-protein-coupled receptors as well (Billadeau, 2008). They rather prefer to migrate towards N-Formylmethionine-leucyl-phenylalanine (fMLP) - a known bacterial chemoattractant - or complement component (C5a), which assists in the removal of pathogens (Billadeau, 2008). For neutrophils to migrate from circulation to the actual site of infection in tissue, they need to respond to various chemokines such as interleukin-8 (IL-8), TNF-α, complement peptides (e.g. C5a and C3a), and leukotriene B4 (LTB4). They also respond to chemicals released directly by bacteria itself such as fMLP (Mondal *et al.*, 2012). All these different signals are needed as they play important roles in different pathways that assist in more effective neutrophil recruitment.

In order to explain neutrophils chemotaxis preference, studies have shown that neutrophils prefer to migrate towards fMLP and C5a, even when their concentrations are significantly lower than that of chemokines and leukotrienes (Foxman *et al*, 1997), (Heit *et al.*, 2002) ; (Billadeau, 2008). Billadeau (2008) noted that fMLP and C5a migration makes use of the mitogen-activated protein kinase (MAPK, p38) while most other chemokines are reliant on phosphatidylinositol-3-OH kinase (PI3K). This may explain why fMLP is often used as chemotaxin of choice in *in vitro* studies. The fact that fMLP – although specifically applicable to pathogen presence – also used the MAPK38 system, makes it a suitable alternative for C5a, which is more broadly applicable to all inflammatory scenarios, but which is more difficult to use given its endogenous presence (Billadeau, 2008).

Returning to the mechanism(s) facilitating chemotaxis preference, Heit *et al.*, (2008) has suggested that neutrophils are able to prioritize chemoattractant signals *via* phosphatase and

tensin homolog (PTEN). Interestingly, PTEN-deficient neutrophils that were migrating towards fMLP became disorientated when CXCL2 (another chemokine, also called macrophage inflammatory protein (MIP) was added, negatively affecting migrational accuracy. Also, when first migrating towards CXCL2, the cells did not change direction when fMLP was added, suggesting PTEN has a major role with regards to migratory decision making with regards to different chemoattractant (Papakonstanti *et al.*, 2007). This mechanism has not been comprehensively investigated. The majority of research into PTEN [as well as Phosphatidylinositol (3, 4, 5)-triphosphate (PIP3) and PI3K] has been focused on their chemotaxis-related roles to facilitate macrophage mobility and directional accuracy *via* macrophage mechanical polarisation. The contribution of these phenomena are discussed in the next section.

2.2.5 Neutrophil polarization

Neutrophils are highly motile and have the ability to interpret and translate any chemoattractant gradient into the polarization of the cell and also the alignment of polarity and chemoattractant gradients to occur (Xu *et al.*, 2010). Neutrophil polarization is the process that causes the neutrophil to undergo a mechanical shape change (elongation), showing a clear leading and tail end of the cell (Shi *et al.*, 2009) (i.e. quite different from macrophage polarisation) in order for actual movement to take place. Polarization of neutrophils is very important in assisting cell movement: actin polymerization at the leading edge pushes the cell forward and actomyosin contractility at the rear end allows tail retraction to occur. At the leading edge there is continuous generation of F-actin meshwork that pushes the cell forward and at the tailing end constant dissolving of previous attachment occurs, this allows the cell to translocate and reattach creating movement (Weiner *et al.*, 1999), (Srinivasan *et al.*, 2003), (Shi *et al.*, 2009).

Polarization assists in faster translocation of the cell, but does not play a role in directionality of the cell during chemotaxis, although abnormalities in the process will affect the ability of the cell to maintain directional accuracy. Various cellular proteins are involved in the complexity of cellular propulsion.

2.2.5.1 PI3K, PIP3 and PTEN

In order for chemotaxis to be effective, both motility and directional accuracy are needed, although the two do not seem to be directly interdependent. The exact contribution of all the key components involved in the leading and trailing edge of neutrophil migration is still not fully discovered, but better understanding is starting to emerge.

Although Xu *et al*., (2003) reported that the chemoattractant signal is solely responsible for directionality during neutrophil migration, this is a somewhat outdated idea. Rather, the chemoattractant might be the initial step which dictates the direction in which the cell would like to go, but a variety of factors have to come into play to help the cell accomplish directionality and motility towards the chemoattractant. For a neutrophil to successfully migrate in the direction of the chemotactic signal, some synchronised events have to occur (**Figure 2.2**). A F-actin meshwork has to be formed in what becomes the leading edge of the cell, to facilitate forward propulsion (Petrie *et al.*, 2009), (Kamakura *et al.*, 2013), while actomyosin contraction has to occur at the trailing edge of the cell for detachment.

Some of the regulators thought to function in the front (leading edge) are phosphatidylinositol 3, 4, 5 –triphosphate (PIP3) and the small GTPases (e.g. Rac, PI3K and Cdc42), while in the rear other factors come into play (e.g. RhoA GTPase, phosphorylated myosin light chain (pMLC) and PTEN (Xu *et al.*, 2010), (Kamakura *et al.*, 2013). Especially the Rho family GTPases are crucial in initiating cell polarization and motility and are relied on strongly for chemotaxis (Srinivasan *et al.*, 2003), (Pestonjamasp *et al.*, 2006).

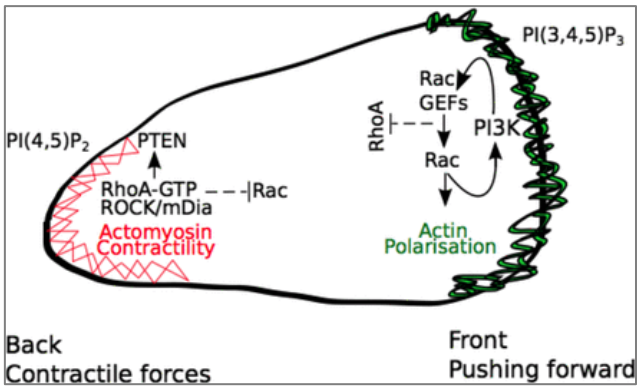


Figure 2.2. Proposed model of neutrophil polarization from: (Gambardella *et al.*, 2013)

As illustrated in Figure 2.2, at the leading edge, PI3K is responsible for synthesis of PIP3 (Billadeau, 2008), (Heit *et al.*, 2008). PIP3 accumulation at the leading edge is seen as an early and noticeable occurrence once cell polarization is initiated as a result of chemoattractant stimulation (Servant *et al.*, 2000), (Hawkins *et al.*, 2010) and has been linked to various cytoskeleton-based functions such as adhesion and migration (Hawkins *et al.*, 2006), (Mondal *et al.*, 2012). In our context, PIP3 is required for the activation of the GTPases Rac (Gambardella *et al.*, 2013) and Cdc42 by Dock2 (a PIP3-dependent guanine nucleotide exchange factor [GEF]) (Wang, 2009); (Berzat *et al.*, 2010). Activated Rac and Cdc42 then signals for the accumulation of F-actin as well as focal inhibition of RhoA in the leading edge, thus effecting forward propulsion (Weiner *et al.*, 2002), (Xu *et al.*, 2003), (Berzat *et al.*, 2010), (Gambardella *et al.*, 2013). This process is sustained by activated Rac, which seems to maintain PIP3 production by PI3K in a positive feedback loop.

Simultaneously, at the rear end of the cell, a contrasting picture is seen: RhoA signalling seems to inhibit Rac activation and thus allows for the recruitment of PTEN to the rear. PTEN further inhibits PI3K (Vemula *et al.*, 2010) and dephosphorylates PIP3 to phosphatidylinositol 4,5–bisphosphate (PIP2) (Gambardella *et al.*, 2013), maintaining Rac in its inactivated form. This role of PTEN has been shown to be vital for the maintenance of directional accuracy (Billadeau, 2008), (Heit *et al.*, 2008). Similarly, another phosphatase – SH2 domain-containing inositol 5-phosphatase (SHIP1) – has been illustrated to have a similar function to PTEN (Mondal *et al.*, 2012). This group has also linked excessive PIP3 at the rear of a migrating cell to suboptimal anterior-posterior PIP3 gradient, which is needed for successful migration. This supports our earlier notion that directional inaccuracy is a result of suboptimal polarisation. Interestingly, the opposing actions on Rac in the front and back edge of the cell seem to occur independently of each other, although in a synchronised fashion. More research is required to more fully elucidate the connection between these occurrences.

In my opinion, using PI3K as a marker for cellular migration would be a better choice for a marker than PIP3. As discussed above, it is the PIP3 gradient between the front and rear end of the cell that is most informative, rather than total concentration. Thus, accurate information can only be gained from image analysis type assessments. On the other hand, PI3K is generally accepted to only be found in the front of the cell, so that for this parameter, total expression would provide sufficient information.

The relative importance of the various role players have been investigated and argued to some extent. For example, disturbance of SHIP1 in neutrophils resulted in F-actin polymerization and PIP3 build-up across the entire cell (Nishio *et al.*, 2007). This was associated with super-adherent neutrophils showing significantly inhibited migratory capacity (Nishio *et al.*, 2007), suggesting importance of SHIP1 in neutrophil polarisation. However, it was noted that a loss of PTEN also allowed PIP3 and F-actin production to continue, which resulted in similarly inefficient chemotaxis (Mondal *et al.*, 2012), arguing for importance of this phosphatase as well. Although much of the molecular mechanisms at play remains to be elucidated, there are some clues in the literature to suggest slightly different roles for PTEN and SHIP1. A study by Mondal *et al*., (2012) illustrated that the loss of SHIP1, but not of PTEN, led to an increase in cellular adhesion. Furthermore, a subsequent study by the same group demonstrated no loss of chemokinetic accuracy in SHIP1-deficient neutrophils toward fMLP (Mondal *et al.*, 2012). Also, SHIP1-/- mice showed an increase in pro-inflammatory cytokine release and neutrophil recruitment (Strassheim *et al.*, 2005), (Nishio *et al.*, 2007), (Mondal *et al.*, 2012). In contrast, neutrophils with experimentally altered PTEN genetics, showed relatively minor abnormalities with regard to cellular migration (Subramanian *et al.*, 2007), (Heit *et al.*, 2008); (Schabbauer *et al.*, 2010). However, these PTEN-compromised neutrophils exhibited decreased directionality of the response towards chemotactic signals (Heit *et al.*, 2008). From this, it can be concluded that SHIP1 probably has the more important role in cell mobility *via* regulation of synchronised detachment and adhesion, while PTEN seems more important for maintenance of directional accuracy.

Most surprisingly, neutrophils lacking any of PIP3-metabolizing enzyme's PI3K, PTEN, SHIP1, or neutrophils insufficient in PIP3 levels as a result of wortmannin treatment (Kamakura et al., 2013), all still showed maintenance of at least some degree of directionality during chemotaxis (Ferguson *et al.*, 2007), (Nishio *et al.*, 2007), (Hoeller *et al.*, 2007). Thus, although the above literature does seem to suggest specific importance for the factors discussed, there remains significant redundancy. This phenomenon testifies once again to the importance of the innate immune system, but also opens the door for further research to more fully elucidate this complex puzzle.

2.2.5.2. ROCK

RhoA/ROCK was mentioned in the above section, but we will be taking a closer look at how the family members of Rho-GTPase plays a vital role in managing certain cellular processes such as migration, proliferation and apoptosis (Shi *et al.*, 2009), (Vemula *et al.*, 2010). The main downstream effector of Rho-GTPase in the rear of the cell has been identified as p160-Rho-associated coil-containing protein kinase (ROCK) (Vemula *et al.*, 2010), (Heasman *et al.*, 2011). Here, Rho-GTPs activates ROCK to assist the actomyosin-mediated contraction and tail retraction that is required for forward movement.

Taking a closer look at ROCK specifically, there are two known isoforms of ROCK, namely ROCK1 and ROCK2, which shares more than 90% homology with their kinase domain (Nakagawa et al., 1996). The exact functional differences between ROCK1 and ROCK2 are not well understood, although the fact that varying levels of ROCK1 and ROCK2 activity throughout the body implies some functional specificity. For example, ROCK1 activity is lower than that of ROCK2 in lung endothelial cells, but higher in fibroblasts (Vemula *et al.*, 2010), although it remains controversial whether ROCK1 and ROCK2 exhibit functional redundancy (Zanin-Zhorov et al., 2016). For example, ROCK1 and ROCK 2 were shown to have similar roles with regard to cell cycle development and tumorigenesis (Kümper *et al.*, 2016) while having specific roles in the modulation of keratinocyte differentiation and cellular tail retraction/detachment (Yoneda *et al.*, 2005). Thus, it seems as if the ROCK1 versus ROCK2 function is dependent on the cellular system where it is expressed.

From the innate immunity's perspective , ROCK1 is thought to be needed for the stress fiber formation and the phosphorylation of the myosin light chain, while ROCK2 on the other hand has a stronger binding capacity to PIP2 than ROCK1 (Vemula *et al.*, 2010). In a study on 8-week old wild type and ROCK1 -/- mice, macrophages and neutrophils were extracted from bone marrow: ROCK1-deficient cells exhibited a 60% decrease in the overall ROCK activity, which was associated with both enhanced cell recruitment and enhanced migration in response to a variety of chemotactic stimuli, which coincided with increased PIP3 levels. ROCK1 deficiency resulted in increased PIP3 and AKT levels, which was ascribed to diminished PTEN activation, although no direct link was seen between PTEN and ROCK1 in the knockout animals. Vemula *et al.* (2010) concluded that ROCK1 is a regulator of PTEN, whose function is to limit excessive recruitment of macrophages and neutrophils during acute inflammation. Vazquez *et al.*, (2001) showed similar results to Vemula *et al.* (2010) that the

loss of PTEN enhanced recruitment of neutrophils during experimental acute peritonitis. This is also in agreement with earlier studies (Papakonstanti *et al.*, 2007) (Subramanian *et al.*, 2007) showing that PI3K activation resulted in ROCK-dependent PTEN inhibition and improved migration towards fMLP.

Additionally, the amount of PIP3 as well as its localization within the cell have been reported to be positively associated with migration/chemotaxis and the recruitment of immune cells (such as macrophages and neutrophils) in response to multiple receptors being activated, as PTEN is a regulator of PIP3 (Vanhaesebroeck *et al.*, 2001); (Hawkins *et al.*, 2006). Furthermore, increased localization of PTEN in the rear of the cell, resulted in better neutrophil migration towards fMLP (Li *et al.*, 2005). This role for PTEN and/or ROCK in phagocyte migration may identify these parameters as potential therapeutic targets with which to modify inflammation. However, it is important to note that the loss of PTEN has been linked to cancer (Billadeau, 2008). Thus, this avenue should be approached with caution. Nevertheless, from the results discussed above, ROCK1 seems to be an important negative regulator of cell recruitment and migration.

In contrast, total ROCK-/- neutrophils have been described as having a tail-retraction defect (Pestonjamasp *et al.*, 2006); (Worthylake *et al.*, 2001), arguing for a positive effect of ROCK on at least cell migration. However, given the lack of ROCK-specificity of the knockout model used, it is difficult to interpret this result. Recently, ROCK1 and ROCK2 were suggested to have opposing effects in macrophage polarisation (Zandi *et al.*, 2015). However, more research is required before conclusions can be made with regard to opposing effects for ROCK isoforms in neutrophils specifically. Given the lack of available literature on ROCK2, ROCK1 is currently probably the better isoform to assess for information on modulation of inflammation.

2.2.5.3 Diaphanous-related Formin-1 (mDia1)

Another known RhoA-GTP effector found in the front of the cell is diaphanous-related formin-1 (mDia1), a protein that has been known to regulate actin rearrangements (Gambardella *et al.*, 2013). mDia1 is responsible for the elongation that occurs at the front of the cell by regulation of the unbranched actin filaments while ROCK is able to control pMLC levels and in return increase actin filament contractibility (**Figure 2.3**) (Gambardella *et al.*,

2013). Shi *et al*., (2009) stated that neutrophils lacking mDia1 are unable to successfully polymerize actin, polarize and maintain directional migration and this is linked to the decreased activation of RhoA-ROCK pathways. mDia1 -/- neutrophils showed decrease in total distance covered, speed and directionality when migrating towards fMLP (Gambardella *et al.*, 2013). ROCK induced regulations as well as the relocalization of phosphorylated MLC (pMLC) which is normally to the rear and sides of the cell was scattered randomly inside the cell resulting in problems with tail retraction and lateral pseudopodia (Shi *et al.*, 2009). It can thus be concluded that the loss of mDia1 serves a role in coupling the chemoattractant-triggered RhoA activation to the neutrophils cytoskeleton, but to also stimulate RhoA-ROCK pathway (Shi *et al.*, 2009).

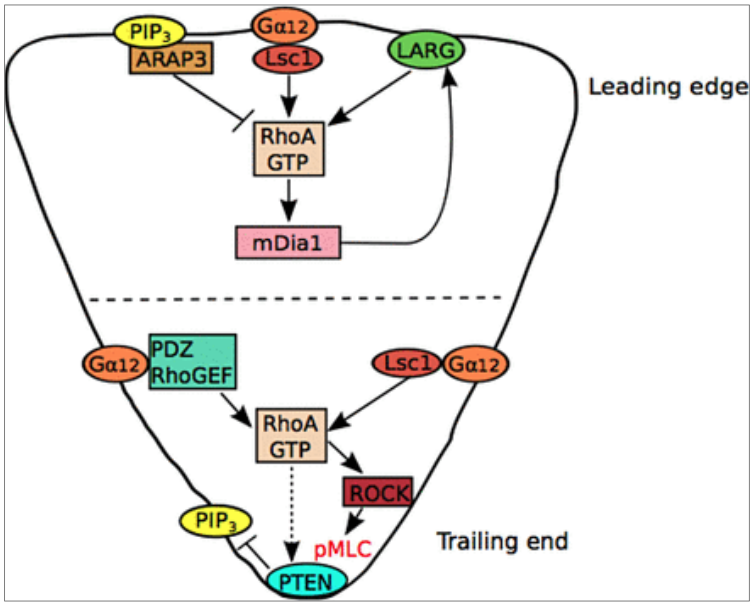


Figure 2.3 Proposed model of RhoA signalling in the neutrophil by (Gambardella *et al.*, 2013).

2.2.5.3 Summary

From the studies discussed, it is clear that the process of chemotactic movement of neutrophils is an extremely complex one. In summary, it has been determined that for chemotaxis to be effective both motility and directional accuracy needs to be maintained and that they are not dependent on each other. Furthermore, polarization of a neutrophils gives a

clear leading and rear edge to the migrating cell, thereby enabling directional movement. Polarization is characterised by a number of synchronised events: F-actin formation at the leading edge that drives the cell forward, tail retraction of the rear which propels the cell forward, as well as maintained cell polarity *via* PIP3-gradient. It is believed that the F-actin meshwork is responsible for directionality, while the maintenance of the cell's front-rear polarity is responsible for motility. The F-actin meshwork is controlled by activated Rac that is regulated by PI3K *via* Rac-GEF and this is what drives the cell forward. Activated Rac also stimulates PI3K, which in turn synthesis PIP3. In some cases it is thought that PIP3 is the driving force and in other cases Rac, but a consensus about the mechanism has been reached to some extent. Both the leading edge and the rear of the cell need to work in a synchronised manner to move the cell forward. The movement of the cell is thought to be achieved by positive feedback loops. The rear of the cell is able to detach from the substratum by RhoA/ROCK-dependent actomyosin contractile forces, which in turn allows a net forward translocation of the cell. During chemotaxis PI3K is localized to the front of the cell and PTEN to the rear of the cell, RhoA signalling inhibits Rac activation and allows the recruitment of PTEN to the rear. The fact that PTEN and PI3K are located in different parts of the cell means the play a pivotal role in the control of PIP3 being distributed to specific compartments. While chemotaxis is underway, an anterior-posterior PIP3 gradient needs to be obtained inside the cell and this can guide the cell into a certain direction, stimulating a type of compass. PIP3 plays a vital role in the stabilization of the front-rear polarity, the regulation of PIP3 *via* PI3K in the front of the cell pushes cell forward, while PIP3 can be down regulated by PTEN to PIP2 in the rear. PTEN is thought to be the main regulator in maintaining the front-back polarity. Excess PIP3 in the rear of the cell could disrupt the PIP3 anterior-posterior gradient and it is needed for successful migration. SHIP1 marker was identified and reported to be found in the rear of the cell and is also able to phosphorylate PIP3 to PIP2. The loss of both PTEN and SHIP1 independently resulted in the same over production of PIP3 and the F-actin meshwork. However only SHIP1 deficient neutrophils showed an increase in cellular adhesion and not PTEN deficient cells. Therefore, PTEN is the main driving force in maintaining the PIP3 gradient between the leading and rear of the cell. While SHIP1 is to be a negative regulator of Ptdlns(3, 4, 5)P3 formation at the rear of the cell, assists in preventing the top-down Ptdlns(3, 4, 5)P3 polarity and lastly SHIP1 facilitates tail attachment and retraction. PTEN is directly associated with chemoattractant signalling *via* GPCR activation, while SHIP1 is involved in the regulation of integrin mediated adhesive responses. PI3K is thus a great marker for determining the expression of PIP3 at the leading

edge during migration. Increase in PI3K at the leading edge should result in an increase of PIP3 at the leading edge and the down regulation of PIP3 at the rear. Rho-GTPase plays a vital role in managing certain cellular processes such as migration, proliferation and apoptosis. RhoA GTP is found in both the front and rear of the cell, and that Rac and Cdc42 are GTPases that promotes the production of F-actin in the leading edge, while Rho-GTPs such as ROCK are located at the rear and assist the actomyosin-mediated contraction and tail retraction that is required for forward movement. ROCK is thought to activate PTEN and in return PTEN phosphorylates PIP3 to PIP2. The loss of ROCK, increases PIP3 and Akt levels because of diminished PTEN activation, which in turn resulted in enhanced migration and enhanced F-action meshwork. This is where the results start to differ as some studies claim the loss of ROCK and PTEN will result in tail retraction problems and reduce neutrophil migration.

2.3 Macrophages

Apart from neutrophils, macrophages also has an important role to play in the inflammatory process. Unlike neutrophils, which are generally accepted to exist as one homogenous population, macrophages can be divided into different phenotypes with somewhat different functions. In this section, a brief overview on the relevant literature on macrophages is presented.

2.3.1 Role of macrophages in inflammation

Monocytes are highly active and versatile mononuclear phagocytes. After circulating in blood for a period of about 20 hours, monocytes differentiate into macrophages, which then leave circulation to enter tissue, where they can remain for the next 20 years, to provide local innate immunity (Geissmann *et al.*, 2010), (Gordon, 2012), (Mia *et al.*, 2014).

Given the huge impact of inflammation in the sporting arena, the majority of research on inflammation and macrophages, in the absence of pathology, has been done with skeletal muscle injury as focus. In this context, macrophages are known to be of extreme importance for tissue repair, forming part of the late inflammatory phase of recovery (Smith *et al.*, 2008).

The involvement of macrophages during the second phase of recovery have been established and are thought to include: clearing of cellular debris (including expended neutrophils) *via* phagocytosis; macrophage phenotypic shift from pro- to anti-inflammatory; inhibition of muscle cell apoptosis; secretion of factors that can assist in muscle precursor cell activation as well as growth; and release of cytokines and growth factors that can promote vascular and muscle repair (Massimino *et al.*, 1997), (Li *et al.*, 2001), (Gordon, 2003), (Chazaud *et al.*, 2003), (Järvinen *et al.*, 2005), (Tidball, 2005), (Sonnet *et al.*, 2006), (Summan *et al.*, 2006); (Arnold *et al.*, 2007), (Smith *et al.*, 2008).

However, in order for them to fulfil these very important roles to prepare damaged sites for tissue regeneration, it is vital for macrophages to migrate either from circulation, or from tissue where they have been residing, to damaged tissue. In the next section, I will review what is known about macrophage phenotyping, as it is a sub-theme in my thesis with regards to whether a phenotypic shift occurs.

2.3.2 Macrophages phenotypes

Macrophages can be classified into four main groups. Firstly, M1 (classically activated) macrophages – the release of which are regulated by TH1 cytokines – are known to secrete pro-inflammatory cytokines as well as ROS and display great microbicidal and tumoricidal properties (Tan *et al.*, 2016). As is the case for neutrophils, ROS in pro-inflammatory macrophages plays an important role in the degradation of ingested material, but in excess, can induce apoptosis of the immune cell itself, which exacerbates (secondary) inflammation (Tan *et al.*, 2016). Alternatively activated macrophages are sub-divided into three sub-populations: M2a macrophages are induced by TH2 cytokines (e.g. IL-4) and are known to secrete various specific markers (e.g. YM1, AMCase and Arginase 1) (Saclier *et al.*, 2013) (Tan *et al.*, 2016). These specific markers are linked to the synthesis of the extracellular matrix, angiogenesis and parasite clearance (Saclier *et al.*, 2013), but are not thought to be present in significant quantities in the absence of chronic pathogenic infection or chronic inflammatory disease (e.g. chronic fibrosis). M2b macrophages are activated by a mixture of toll-like receptor ligands and immune complexes to dampen inflammation (Saclier *et al.*, 2013). A literature search did not reveal many studies focusing on this particular phenotype, so that its relevance is uncertain at this point. Finally, and of specific relevance in the context

of inflammation, M2c macrophages produce high levels of anti-inflammatory proteins, such as IL-10 and transforming growth factor-beta (TGF-β) (Saclier *et al.*, 2013), (Tan *et al.*, 2016). Both IL-10 and TGF-β are known to assist the cell in expressing cell surface markers like mannose receptor (MR/CD206) and scavenger receptor (SR/CD163) (Tan *et al.*, 2016). M2 macrophages can be grouped by stimulating the cells with either IL-4, IL3, TGFβ or IL-10 which in turn showed different distinctive activation profiles (Mantovani *et al.*, 2004). This is done as there are many overlapping characteristics in the alternative M2 macrophage phenotypes apart from the IL-4 induced phenotype (M2a) (Tan *et al.*, 2016).

However, instead of existing as 4 distinct macrophage cell types, these cells seem to be just different phenotypes arising from one cell type only. The surrounding cellular environment seems to change macrophage phenotype by polarizing them, allowing them to perform their specific functions (Geissmann *et al.*, 2010). The specific time-order of these different macrophage phenotypes has been defined, at least in the context of non-pathological tissue damage. Here, the first cells present at the site of injury are the pro-inflammatory M1 macrophages, which seem to fulfil a function similar to that of neutrophils. After a few days they undergo a phenotype shift to become anti-inflammatory M2 macrophages (Arnold *et al.*, 2007), to facilitate resolution of inflammation. Interestingly, increased population of pro-inflammatory macrophages has been reported in individuals suffering from autoimmune diseases ,(Kouwenhoven *et al.*, 2001), (Menke *et al.*, 2009), (Li *et al.*, 2010), (Ciccia *et al.*, 2013), suggesting that a predominant macrophage phenotype may play an important role in modulation of inflammation, and thus perhaps cell types other than macrophages involved in the process.

In support of this, manipulation of this phenotype shift has been proposed as therapeutic avenue and has been investigated to some extent. Kruger *et al.*, (2012), showed that the use of an antioxidant treatment had beneficial effects on the resolution of inflammation and one of the ways it does this, is by facilitating a phenotype shift from M1 to M2 macrophages (this will be discussed later in more detail in section 4). Mia *et al.*, (2014) similarly suggested that prolonged persistence of pro-inflammatory macrophages may lead to the prolongation of inflammation resolution. This group of researchers illustrated how human peripheral monocytes from both healthy and diseased (multiple sclerosis or spondylarthritis) donors may be polarised with either lipopolysaccharides (LPS) and IFN-γ, or IL-4, IL-1β and TGFβ, to yield M1 and M2 macrophage phenotypes respectively. This follows earlier work by the same group in models of autoimmune disease, where autologous infusion of pre-activated

anti-inflammatory M2 macrophages assisted in reducing pro-inflammatory M1 expression (Parsa *et al.*, 2012), (Wållberg *et al.*, 2005). Together, these studies point to manipulation on the macrophage phenotype as a viable target for therapeutic effect.

Unfortunately, phenotyping of macrophages has been known to be quite the challenge. Currently, the convention for human macrophages is to classify M1 macrophages as those with high expression of CD86, CD274 and HLA-DR, while M2 macrophages exhibit high expression levels for CD206, CD163 and intracellular IL-10. It is important to note that this classification was derived from the experimental polarisation of *in vitro* cell populations. However, classification of cells isolated from whole blood is not a simple sorting process. Using the same six markers, Saclier *et al*., (2012) illustrated that many macrophage phenotypes were present in the same area at the same time, suggesting that the chronology of macrophage phenotype switch is not as uniform and synchronised unless subjected to a uniform, high-concentration stimulus, as employed in the polarisation protocols. Also, phenotype switching basically entails lowering of expression of some markers and increasing expression or others. Thus, since any primary macrophage population will consist of many cells in transition, much variation in terms of expression levels of all six markers is exhibited and no clear-cut phenotype categories can be distinguished, unless the stimulus for phenotype shift is of extreme magnitude/severity. This interpretation is in line with the opinion of the Mantovani group – an authority on macrophage phenotyping – who suggested that M1 and M2 macrophage phenotypes are extremes in a continuum of polarisation states (Sica *et al.*, 2012). For this reason, the norm has become to investigate changes in phenotype shifts by stimulation of primary cell samples *in vitro* prior to typing (Mia *et al.*, 2014). Much more work is needed on this topic, given both its complexity and its potential as target for intervention.

Finally, in the context of this thesis, a phenotype switch in the periphery – e.g. as a result of supplementation – may affect the cytokine environment of the neutrophil, with predominance of pro-inflammatory M1 (and M2a in chronic inflammation specifically) cells creating a relatively pro-inflammatory environment, while M2c predominance may result in a more anti-inflammatory environment. Thus, the phenotype of macrophage may affect neutrophil activation and recruitment into tissue. It is thus important to consider changes in both cell types, and not just neutrophils.

This concludes the review on the general functioning of the innate immune system in the context of inflammation. Given the focus of the thesis – investigation of the modulatory

effect of grape seed-derived polyphenols on inflammation – I will now switch to an overview of literature related to this natural medicine.

2.4 Polyphenols

Triterpenoids and polyphenols are plant-derived compounds that are known to have anti-oxidative capacity and are able to some degree to provide alleviation against inflammatory stress (Dinkova-Kostova *et al.*, 2005), (Morillas-Ruiz *et al.*, 2006). The clinical significance of the anti-inflammatory capacity that polyphenols possess are still being debated. A few studies done on polyphenols have helped to shed some light on this issue.

The common saying "too much of a good thing becomes a bad thing", is applicable when it comes to oxidants and inflammatory processes. When the production of oxidants is far greater than the endogenous antioxidant capacity, oxidative stress arises (Petersen *et al.*, 2016). As discussed previously, inflammation plays a vital role in fighting off infection and tissue repair but also causes secondary damage. As recently reviewed (Petersen *et al.*, 2016), the inflammatory immune response and oxidative stress are interlinked processes. Therefore therapeutic treatments that can alleviate the cumulative effects of both are being investigated. Here, an overview of a variety of plant extracts or plant-derived compounds and polyphenols that have been proposed to have anti-inflammatory and/or antioxidant effect is provided.

In the context of cardiovascular disease, much research has focused on modulatory effects of polyphenols. For example, quercetin and resveratrol, both polyphenols contained in red wine, were shown to interfere with pro-inflammatory nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) signalling of thrombin by preventing adenosine nucleotide secretion from activated platelets (Kaneider *et al.*, 2004). This is a significant finding, as thrombin is thought to play a role in facilitating inflammation and reparative responses to vascular injury (Ruf *et al.*, 2003), while thrombin-related down-regulation of endothelial ectonucleotidase activity results in high adenosine diphosphate (ADP) and adenosine triphosphate (ATP) levels, which in return leads to the activation of platelets, leukocytes and endothelia (Daniel *et al.*, 1999), (Kaneider *et al.*, 2004). Thrombin-activated platelets are known to cause an increase in neutrophil respiratory bursts, but resveratrol and quercetin was found to inhibit these bursts, as well as neutrophil migration towards conditioned media from platelet cultures exposed to thrombin (Kaneider *et al.*, 2004). Interestingly, these

polyphenols, such as epigallocatechin 3-gallate (EGCG), which is the major polyphenol present in green tea was seen prevent endothelial dysfunction and inhibit NF-κB activation (Ahmad, Gupta and Mukhtar, 2000). This was seen when A431 cancer cells were exposed to an inflammatory stimuli, such as LPS by disturbing the Rho-GTPase-dependent signalling pathways (Chen *et al.*, 1996), (Ahmad *et al.*, 2000), (Balasubramanian *et al.*, 2002).

Similarly, vegetables high in dietary fibre have been linked to a decrease in the prevalence of coronary heart disease (CHD), stroke and cardiovascular mortality (Gaziano *et al.*, 1995), (Li *et al.*, 2001), (He *et al.*, 2006). A large group of vegetables of the Brassicaceae family – including broccoli, cauliflower, sprouts and kale are known for their anti-oxidative and anti-inflammatory capacity and therefore have been studied within the last decade with great interest (Kuntz *et al.*, 2014). Brassicaecae has a significantly high content glucosinolates, polyphenols and phenolic acids (Cartea *et al.*, 2010), (Velasco *et al.*, 2011). Studies in humans have shown that an atherogenic diet's negative effects can be prevented by consumption of *Brassica oleracea L.* (Noh *et al.*, 2009), (Sankhari *et al.*, 2012). Furthermore, *in vitro* studies elucidated certain particulates in Brassicaceae were able to lower the level of cellular adhesion molecule expression, which are known role players in inflammation and atherogenesis (Chao *et al.*, 2013).

Of particular interest and relevant to the specific topic of this thesis is the adhesion of cultured neutrophils to TNF-α-stimulated endothelial cells that decreased from 57% to 36% after pre-incubation of methanolic and water extracts of curly kale leaves, while adhesion to non-stimulated endothelial cells was not affected (Kuntz *et al.*, 2014). In addition, in the same study, TNF-α-induced increased expression of VCAM, ICAM and E-selectin mRNA was attenuated after treatment. Similar results was also reported for kaempferol-3-O-sophoroside, another flavonoid found in high amounts in cabbage (Kim *et al.*, 2011). These effects were ascribed to potential modulation of the redox NF-κB signalling pathway – similar to the mechanism suggested for quercetin and resveratrol in the context of thrombin.

Also in the context of cancer, Brassicaceae flavonoids – specifically the polyphenols – were shown to have similar beneficial effects *via* inhibition of TNF-α induced ICAM-1 and VACM-1 expression in different cancer cell lines (Chen *et al.*, 2004); (Jung *et al.*, 2012); (Jang *et al.*, 2012). The authors suggested that the beneficial effects were due to their anti-oxidant capacity. Additionally, a study by (Engelbrecht *et al.*, 2007) using grape seed proanthocyanidin extract (GSPE) on colon cancer cells (CaCo2 cells) to assess its chemo-preventative/anti-proliferative capacity showed that an increase in apoptosis was associated

with inhibited PI3K expression and decreased PKB SER^{473} phosphorylation (Engelbrecht *et al.*, 2007).

Curcumin is another well-known polyphenol, which is found in turmeric curry spice. It gives the yellow colour we are familiar with and is also known to possess anti-inflammatory, anti-oxidative, anti-proliferation and anti-angiogenic activities. For example, curcumin down-regulated the adherence of neutrophils to HMGB1-activated endothelial cells and their migration towards chemotactic fMLP in concentration dependent manner. Furthermore, curcumin attenuated HMGB1-mediated increased in VCAM-1, ICAM-1 and E-selectin expression (Kim *et al.*, 2011). High mobility group box 1 (HMGB1) protein up-regulates pro-inflammatory cytokines in several inflammatory diseases (Kim *et al*, 2011) and increased extracellular HMGB1 accumulation is indicative of cell or tissue damage (DeMarco *et al.*, 2005), (Erlandsson-Harris *et al.*, 2006).

From these studies, it is clear that the systems modulated by polyphenols are inflammation and oxidative stress, i.e. not disease-specific mechanisms. Several studies have illustrated the interlinked nature of inflammation and antioxidants in the context of plant medicine. For example, Guabiju extracts have antioxidant properties and are able to prevent neutrophil chemotaxis towards lipopolysaccharides. Similarly, quercetin, another plant derived antioxidant is known to also have anti-inflammatory properties (Andrade *et al.*, 2011) (Derlindati *et al.*, 2012). Thus, the modulation by these systems by polyphenols, particularly as preventative therapy, is gaining popularity with end users and researchers alike. One of the most comprehensively studied natural polyphenols, apart from resveratrol and quercetin, is the proanthocyanidins, which are also derived from grapes and which is the intervention chosen for this thesis. This is the focus in the next section.

2.4.1 Grape-derived polyphenols

As mentioned, the inflammatory process consists of the removal of debris and the activation of the cytokine facilitated process that assists in the regeneration (Farges *et al.*, 2002); (Hodgetts *et al.*, 2006). However, it is also known that phagocytes, and in particular neutrophils, causes damage to otherwise healthy tissue in the process. Inhibition of neutrophil oxidative burst has for example been shown to reduce inflammatory secondary damage by more than 80% in a model of ischaemia-reperfusion injury in mice (Tidball, 2005). Given the

requirement for inflammation for pathogen destruction, as well as in preparation for tissue repair, global inhibition of the process is not an option. Therefore, a more targeted approach is required. Several research groups have investigated clinical effects of grape-derived products, but only a few focused on specific potential therapeutic cellular targets.

Proanthocyanidolic oligomers (PCO) is a group of polyphenols commonly derived from grapes. PCO has proven to be beneficial in the inflammatory process, using both *in vivo* and *in vitro* models, as well as chronic and acute supplementation protocols. Our research group has been investigating physiological benefits of grape seed-derived proanthocyanidolic oligomers (PCO) for a number of years, particularly in the context of inflammation and muscle damage. The data available has shown that *in vivo* PCO treatment hastens skeletal muscle recovery from injury in rats *via* modulation of the inflammatory response (Myburgh *et al.*, 2012), (Kruger *et al.*, 2012), (Kruger *et al.*, 2014). Two main mechanisms of PCO have been illustrated in rats; firstly, PCO supplemented rats showed a significant decrease in the amount of neutrophil infiltration to the site of experimental, standardised and controlled skeletal muscle injury – a phenomenon well-known to decrease secondary damage (Tidball, 2005). Secondly, supplemented rats showed earlier macrophage migration towards the injured tissue as well as faster resolution of inflammation (Kruger *et al.*, 2012).

Subsequently, these previous studies have shown that *in vivo* PCO supplementation allows classically activated (M1) macrophages to switch to an anti- inflammatory phenotype, named alternatively activated (M2c) at an earlier time point after injury and even while still in circulation (Kruger *et al.*, 2014). Such a change is much desired in the context of inflammation, since M2 macrophages cannot cross endothelial membranes (Kalkman *et al.*, 2016) and will thus not enter tissue, which could explain the faster resolution of inflammation.

In terms of human studies, there is no or very little literature available. Preliminary data from a study by our research group, in humans subjected to plyometric exercise, suggest that the same benefits may be achieved in human models of mild injury. However, this data is not conclusive due to the relatively small-scale injury characteristic of this model, which may mask some effects. However, recent *in vitro* studies using human cells *ex vivo* or human cell line models have shown positive results. For example, in an ongoing study, there is data proving neutrophil chemokinesis is positively affected by *in vitro* supplementation of human leukocytes with PCO (Petersen *et al.*, 2016). The treated cells exhibit more directed movement, suggesting the likelihood of early-phase inflammation facilitated by neutrophils

to be more effective and thus shorter in duration. Based on these findings, we therefore believe that the PCO product – which is commercially available - is not only safe for human consumption, but also beneficial to general health.

Given these promising effects of PCO treatment, the investigation of potential mechanisms by which these anti-inflammatory effects are achieved was undertaken. The anti-inflammatory potential of grape seed-derived PCO in this context was assessed.

Both chronic preventative and acute post-injury PCO treatment in rats subjected to experimental skeletal muscle contusion injury was shown to effectively limit neutrophil migration into the damaged site. This is associated with faster muscle stem cell recruitment and faster tissue repair (Myburgh *et al.*, 2012), (Kruger *et al.*, 2012). Also, in this model, PCO facilitated an earlier macrophage phenotype switch from pro- to anti-inflammatory phenotypes (Kruger *et al.*, 2014), as well as an earlier anti-inflammatory shift in the cytokine profile both in muscle and circulation (Myburgh *et al.*, 2012). Specifically, injury-induced increased levels of TNF-α – a pro-M1 signal – decreased sooner in the PCO-treated animals, while IL-10 – a pro-M2 signal – increased earlier. This is in agreement with a study including resveratrol, which reported an inhibition of pro-inflammatory cytokine (IL-6 and IL-8), as well as down-regulation of activated neutrophils (Donnelly *et al.*, 2004), (Kruger *et al.*, 2014);. In terms of the molecular targets of PCO, from these results it is not clear whether macrophages were directly influenced or rather indirectly due to the downstream result of the limited neutrophil infiltration and associated damage. However, the results clearly indicate that either the mobilization or the extravasation capacity of neutrophils are impacted by PCO. The interpretation of these results is supported by another study which has shown that proanthocyanidin supplementation inhibits TNF-α and cytokine-induced neutrophil chemoattractant (CINC-1) levels in the pleural exudates in a rat model of carrageean-induced inflammation (Garbacki *et al.*, 2004). Suggesting once again the involvement of proanthocyanidin in hindering neutrophil migration.

In addition to the potent anti-inflammatory effect of PCO supplementation, our research group has been at the forefront of elucidating the increase in antioxidant status in skeletal muscle tissue after PCO treatment (Myburgh *et al.*, 2012). MPO levels were inversely correlated with the decrease in the amount of neutrophils present at day 1 post injury (Myburgh *et al.*, 2012). This clearly illustrated the interlinked nature of these responses, as well as interventions aimed at modulation of either of the two. Additionally, another study demonstrating a dose dependent ability of GSPE to scavenge free radicals *in vivo* and *in vitro*

reported that GSPE was significantly better than vitamins C, E and β-carotene at scavenging free radicals and preventing free radical-induced lipid peroxidation, as well as DNA damage (Bagchi *et al.*, 2000).

A factor that has not been previously investigated in regards to PCO has been employed in this study: its effect on adhesion molecules. However, the investigation of adhesion molecules have been included in the above mentioned groups' previous research. For example, one study on human umbilical vein endothelial cells (HUVECs) reported that TNF-α-stimulated VCAM-1 expression was dampened by grape-derived proanthocyanidin treatment (Sen *et al.*, 2001). Similar results were reported for Activin, a new generation antioxidant which is also a grape seed derived proanthocyanidin (Kalfin *et al.*, 2002).

The study performed by (Kalfin *et al.*, 2002) assessed the effectiveness of Activin to decrease the modulation of adhesion molecules in plasma (as an effect of an inflammatory response) from patients suffering from systemic sclerosis (SSc) was investigated. Briefly, patients were orally supplemented with 100mg/day of Activin for one month – this was associated with a significant reduction in the disease-related increased adhesion molecule (VCAM, ICAM, E-selectin) expression in plasma (Kalfin *et al.*, 2002). Also, malondialdehyde (a well know oxidative stress marker) was significantly reduced in SSc patients after Activin treatment (Kalfin *et al.*, 2002).

From these previous studies, it is clear that grape seed derived PCO has both anti-inflammatory and anti-oxidative benefits. Although only a few parameters have been identified which consistently indicate positive effects, the exact mechanism(s) involved has not been comprehensively elucidated. Additionally, few studies involving the supplementation of humans with PCO have been done and there are no previous studies investigating the exact mechanism of neutrophil chemotaxis. Since the modulation of inflammation is not only relevant to individuals in the sporting arena or aged individuals, but also to the aetiology and thus treatment of various chronic disease states, a more complete understanding of the mechanisms affected by PCO is of high relevance to society as a whole.

2.5 HYPOTHESIS AND AIMS

Hypothesis

Before clinical trials may be undertaken in a pathological or at least compromised population, experimental treatments are subjected to testing in healthy individuals. Given the relatively non-extreme anti-oxidant and anti-inflammatory effects reported for PCO in the literature, we hypothesised the following:

- That PCO will be safe for consumption in terms of blood coagulation
- That few or no statistically significant clinical effects of PCO will be evident in a population without a basal inflammatory condition, but that PCO will affect molecular targets which may alter neutrophil chemokinesis

Aims

- To assess effect of 2-week PCO consumption on blood coagulation function
- To elucidate molecular targets of PCO in neutrophils, which may explain PCO-associated differences in neutrophil chemokinesis
- To confirm PCO-associated macrophage phenotype shift in human model

3. CHAPTER 3: METHODS AND MATERIALS

3.1 Study design and ethical consideration

To elucidate cellular mechanisms that may contribute to the polyphenol-induced effects on neutrophil chemokinesis previously reported, a longitudinal, placebo-controlled, double-blind, human *in vivo* study design was chosen. Ethical clearance for all protocols was obtained from the Stellenbosch University Subcommittee C Human Research Ethics Committee (8th January 2016, M15/09/040) prior to subject recruitment. The study was conducted according to the SA MRC ethical guidelines, guidelines for good clinical practice and the International Declaration of Helsinki. (See Addendum I)

3.2 Subject recruitment

Both males and females between the ages of 18-25 years of age were recruited. Recruitment took place by advertisements on campus notice boards. A total number of 18 healthy individuals were selected based on inclusion and exclusion criteria. Inclusion criteria included any person aged 18 to 25 that apparently healthy, not on any type of supplement, recreationally active and non-smoking. Exclusion criteria excluded individuals with known inflammatory disease, chronic disease, or currently suffering from a condition with known inflammatory component, such as sinus or hayfever, from the study. Subjects were instructed to refrain from taking vitamin, anti-oxidant and anti-inflammatory products and not permitted to perform any strenuous exercise for a period of 14 days prior to initiation of the protocol, as well as for the duration of the entire protocol. All subjects were required to refrain from all types of exercise for a period of 24 hours prior to each sample collection time point. (See Addendum H)

3.3 Protocol outline

All volunteers were instructed to read, ask questions and sign their consent forms before enrolment into the study. After the 2-week pre-study wash out phase, participants were given detailed instructions, including a schedule for their three required laboratory visits. Subjects were required to report to the Exercise laboratory (Department of Physiological Sciences, Stellenbosch University) on days 0 and 14 for baseline and post-intervention follow-up, as

well as to Pathcare Laboratories (4 Saffraan Avenue, Stellenbosch) on day 7. (In order to increase compliance to the protocol, participants were reminded of their various appointment dates and times for the duration of the study. Participants were also reminded of the various do's and do not's prior to the study.

On each of these occasions, blood samples were obtained by venepuncture from a forearm vein, by an experienced and qualified phlebotomist. On days 0 and 14, 8ml heparin blood and 8ml ethylenediaminetetraacetic acid (EDTA) blood was obtained from each patient, for assessment. The samples were collected at room temperature and centrifuged immediately after all samples were collected. 8ml heparin blood was used for the neutrophil migration assay, immunohistochemistry staining as well as flow cytometry analysis on neutrophil adhesion markers. While 8ml EDTA blood was used for flow cytometry analysis on macrophage phenotype markers. On day 7, a 2ml sodium citrate anti-coagulated blood sample was obtained, for the assessment of coagulation status, which was included as safety measure only. (See Addendum A)

3.4 Intervention

The grape seed-derived polyphenol used was analysed an independent laboratory (LC-MS laboratory, Central Analytical Facility, Stellenbosch University) to be a proanthocyanidolic oligomer made up of catechin, epicatechin and proanthocyananidin B). Accoring to the supplier's Certificate of Anaylsis, the PCO content of Oxipoven ™ is 70mg per capsule. The daily recommended dosage is 1-2 capsules with water after a meals. Participants were instructed to take two capsules, once a day for 14 days, starting on day 0 (i.e. after baseline blood draw). And after a two week wash out period.

3.5. Sample analysis

3.5.1. Monocyte isolation for flow cytometry

Blood from two EDTA vacationers were used for monocyte isolation. Buffy coats contain all circulating leukocytes, together with residual erythrocytes and platelets, this was layered onto Histopaque 1.077g/ml (sigma-Aldrich, #10771) at 2:1 ratio and centrifuged at 400 x g for

30min at 23°C, without brake. The Erythrocytes and Peripheral Blood Mononuclear Cells (PBMCs) were collected with a plastic Pasteur pipette and washed twice in PBS-EDTA (1x Phosphate Buffered Saline containing 0,5 M Ethylenediaminetetraacetic acid) (Sigma-Aldrich, #P4417 + #E9884) and centrifuged at 300 x g for 10 min at 23°C. Purified PBMCs were resuspended in RPMI 1640 without phenol red (Sigma-Aldrich, #R0883), containing 10% FBS (Fetal Bovine Serum) (Sigma-Aldrich, #12003C) and layered onto a 42.56% iso-osmotic 1.131 g/ml percoll (Sigma-Aldrich, #E0414) solution at a 1.25:1 ratio. The iso-osmotic percoll solution consisted of 48.6% advanced RPMI 1640 with phenol red (Life Technologies, #12633-020), 42.56% percoll, 5.4% FBS and 3.44% 10x PBS. Centrifugation at 550 x g for 30 min at 23°C, without brake and allowed for the separation of monocytes from PBMCs to occur. The white ring containing monocytes was collected with a plastic Pasteur pipette and washed once in PBS-EDTA at 400 x g for 10 min at 23°C, without brake.

Pellet was resuspended in 1ml Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD bioscience #554714) and kept in the fridge at 4°C for 20min to allow for the fixation of cells. After fixation, cells were centrifuged at 400 x g for 5 min at 4 °C. Cells were resuspended in 1ml cytoperm/wash, (BD bioscience #554714) and centrifuged under the same conditions. Cells were resuspended in PBS and aliquotted into different reaction vials (one for "unstained" counting and one "stained"). The 7 antibodies required for monocyte phenotyping BD – Anti-CD86 APC (#555660), BD – Anti-CD163 Per Cp-Cy5.5 (#563887), BD - IL-10 BV 421-Viral HU (#564053), BD - Anti-HLA-DR SPC-Cy7, CE (#335831), BD – Anti-CD206 BB515 (#564668), BD-Anti Myeloperoxidase, MPO, PE (#341642) BD-Anti-CD274 PE-Cy7 (#558017) were added to the "stained" reaction vial. Thereafter, all vials were closed with tin foil and placed in the incubator for 10 min, 95% $CO_2 and$ 5% N_2 to allow for cell labelling. This was followed by centrifugation at 1600 rpm for 5 min at room temperature, resuspended in 1x PBS and overnight storage in the dark, at 4°C. Flow cytometry analysis was performed the next day. (See Addendum B)

3.5.2 Neutrophil Isolation for Flow Cytometry and Migration

Blood from two Heparin vacationers were used for Neutrophil isolation. 4ml heparin anti-coagulated whole blood was layered onto Histopaque 1.077g/ml (Sigma-Aldrich, #10771) at 2:1 ratio and centrifuged at 1800rpm for 30min at 23°C, without brake. Four different layers

of blood were formed, the sediment containing neutrophils and erythrocytes was taken by removing the supernatant. Sediment was mixed with equal volume of 6% Filtered Dextran (6g Dextran and 50ml 1x PBS) (1x Phosphate Buffered Saline, Sigma-Aldrich, #P44170) at room temperature and incubated at 37°C, 95% $CO_2 and\ 5\%\ N_2$ for 45min, to allow sedimentation of erythrocytes. Neutrophil rich supernatant resuspended in 1x PBS and centrifuged at 290xg for 10min at 4°C, this step was repeated. Cells were resuspended in RPMI and an automated cell count was performed on (CellDyne 3700Cs, Abbott Diagnostics; Germany). The final neutrophil pellet was resuspended in the desired volume of RPMI to achieve the cell concentration of +/- $1x10^6$cellls/ml. (Protocol Continues 3.5.5)

For Flow Cytometry preparation - half of the neutrophils isolated were used or this procedure. With a cell concentration of $1x10^6$cells/ml, samples were centrifuged at 1500rpm for 10min at 4°C. Pellet resuspended in PBS (1x Phosphate Buffered Saline, Sigma-Aldrich, #P44170). The neutrophils were aliquotted into different reaction vials (one for "unstained" counting and one "stained"). Surface marker antibody BD – Anti-CD66B FITC (#555724) was added to the "stained" reaction vial and placed in the fridge for 30min at 4°C, covered tinfoil. 1x PBS(1x Phosphate Buffered Saline, Sigma-Aldrich, #P44170) was added and centrifuged at 1800rpm for 6min at 23°C.

Cells were resuspended in Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD bioscience #554714) and placed in the fridge for 25min at 4°C covered in tinfoil. After cell Fixation, cells were washed with cytoperm wash (BD bioscience #554714) and centrifuged at 1800rpm for 6min. Cells were then resuspended in PBS and BD–Anti-CD106, (VCAM-1)PerCP-Cy5.5(#563525) and BD–Anti-CD54, (ICAM-1) BV421 (#566262) was added to the "stained" reaction vial and placed in the fridge for 30min at 4°C, covered tinfoil. Cells were washed with cytoperm wash (BD bioscience #554714) and centrifuged at 1800rpm for 6min. Cell were resuspended in 500ul 4% paraformaldehyde and placed in the fridge overnight at 4°C. Flow cytometry analysis was performed the next day. (See Addendum C and D)

3.5.3 Macrophage phenotype flow cytometry analysis

Macrophages were isolated and single and multiple stain solutions were prepared a day prior for flow cytometry analysis (D FACSAria cell sorter, Becton Dickinson, USA), using D

FACSDiVa v6.1.3 software to determine the Median Fluorescent Intensity (MFI) of each marker. Populations were filtered, relocated and to flow cytometry tubes and immediately analysed. Unstained samples were prepared to determine background fluorescence. Single stain samples (containing only one marker) were used for fluorescent signal optimisation and compensation for potential spill-over between fluorophores. Multiple stain samples were used for data collection and labelled with the following markers: M1 [Anti-CD86 APC (#555660), BD-Anti Myeloperoxidase, MPO, PE (#341642), BD-Anti-CD274 PE-Cy7 (#558017), BD - Anti-HLA-DR SPC-Cy7, CE (#335831)]and M2[BD – Anti-CD163 Per Cp-Cy5.5 (#563887), BD – Anti-CD206 BB515 (#564668), BD-Anti-CD274 PE-Cy7 (#558017)]. The appropriate lasers were sued to excite fluorophores and emission was captured using appropriate band filters. Data collection was done by recording $3x10^5$ events for every donor in triplicate and thresholds were set up by recording $8x10^6$ events. Technical flow cytometry procedures were done by a trained staff member of the Central Analytical Facility (CAF) at Stellenbosch University, however I did undergo basic training and was able to perform my own analyses, but due to time constraints of my study this was not always possible. A macrophage M1 and M2 markers at basal levels were established on day 0 and day 14 for both placebo and active to determine the macrophage phenotype profile of each participant. This would indicate whether PCO treatment provided a phenotypic shift.

3.5.4 Neutrophil adhesion flow cytometry analysis

Neutrophils were isolated and single and multiple stain solutions were prepared a day prior for flow cytometry analysis (D FACSAria cell sorter, Becton Dickinson, USA), using D FACSDiVa v6.1.3 software to determine the Median Fluorescent Intensity (MFI) of each marker. Populations were filtered, relocated and to flow cytometry tubes and immediately analysed. Unstained samples were prepared to determine background fluorescence. Single stain samples (containing only one marker) were used for fluorescent signal optimisation and compensation for potential spill-over between fluorophores. Multiple stain samples were used for data collection and labelled with the following markers: BD – Anti-CD66b FITC (#555724) BD–Anti-CD106, (VCAM)PerCP-Cy5.5(#563525) and BD–Anti-CD54, (ICAM) BV421 (#566262)

The appropriate lasers were sued to excite fluorophores and emission was captured using appropriate band filters. Data collection was done by recording $3x10^5$ events for every donor in triplicate and thresholds were set up by recording $8x10^6$ events. Technical flow cytometry procedures were done by a trained staff member of the Central Analytical Facility (CAF) at Stellenbosch University, however I did undergo basic training and was able to perform my own analyses, but due to time constraints of my study this was not always possible. A neutrophil cellular adhesion marker's expression at basal levels were established on day 0 and day 14 for both placebo and active participants (See Addendum C)

3.5.5 Chemotactic neutrophil migration assay

For migration- half the neutrophils that were isolated were used for migration. Normally, *in vitro* neutrophil migration assays are performed in a Dunn chamber (Sapey *et al.*, 2011), (Sapey *et al.*, 2014) or by making use of microfluidics systems (Irimia *et al.*, 2006), (Irimia, 2010), while videomicroscopy in commonly used for *in vivo* investigations (Germain *et al.*, 2008). However, none of these models allowed for immunostaining of cells at the endpoint, as they are all closed systems. Thus, mechanisms cannot be directly linked to functional outcome. Thus, for the purposes of this thesis, a novel migration assay was developed, by making used of an 8-chamber slide 75ul of $1x10^6$cellls/ml (neutrophils) were seeded to the bottom left hand corner of an 8-well plate. 75ul fMLP [100ug fMLP (Sigma-Aldrich, #F3506) solution with HBSS (Hanks Buffer Solution, ThermoFisher Scientific, #24020091)] or instead 75ul RPMI 1640 with phenol red (Life Technologies, #12633-020) was plated in the top right hand corner, (each well had a duplicate and a control that was replaced with RPMI instead of fMLP. The neutrophil migratory paths were visualized by combining time lapse, images were acquired every 3 seconds per well for 40minutes and taken at 20x magnification using an Olympus Cell system IX-81 inverted fluorescent microscope system with an F-view cooled CDC camera (Soft Imagining Systems) at 37°C. Image J (Java Software) was used to track the movement of Neutrophils and, measure distance covered as well as distance from start using the Mtrack J plugin. Neitrophil tracks were quantified by measuring the total distance they moved (from point to point) and linear distance they covered (straight path from start to finish).

3.5.6 Immunohistochemistry

After live cell tracking, 75ul 4% (v/v) paraformaldehyde in PBS and 75ul RPMI-1640 with phenol red (Life Technologies, #12633-020) was added into each well, to fixate cells and then incubated at 37°C 95% $CO_2 and$ 5% N_2 for 10min. After 10min excess media was removed and wells were washed with 1x PBS (1x Phosphate Buffered Saline, Sigma-Aldrich, #P44170). Cells were then permeabilized with 0.1% Triton X-100 for 5min. Wells were washed with 1x PBS again, 1% BSA (Bovine Serum Albumin Fraction V, Roche #10735078011) (1x PBS) was used as a blocking solution for 20min. Primary conjugated antibodies (Anti-ROCK1, Alexa Fluor 488 #AB205432 and Anti-PI3K #AB202668 Biocom Biotech) were added and left covered in tinfoil overnight in the fridge at 4°C. The next day, wells were washed with 1x PBS. 1% Dappi (20ul Dappi in 2ml 1x PBS), a blue nuclei staining marker was added for 10min. Wells were then washed with 1x PBS. Cells were ready for visualization on the Carl Zeiss LSM780 confocal microscope with ELYRA S.1 Superresolustion platform (Carl Zeiss, Germany) using ZEN Lite 2012 imagiging software (blue and black version). Images (between 1-2) were taken per well in the desired area of chemotaxis (point for contact with chemotaxin or RPMI) and this allowed for qualitative and quantitative analysis. Taking images of one 8-well plate can take up to one hour. This was done by a trained staff member of the Central Analytical Facility (CAF) at Stellenbosch University (See Addendum E)

3.5.7 Immunohistochemistry analysis

Using ZEN Lite (version 2012 blue and black) the images taken could be analysed. Images were uploaded, and analysed one at a time. One image has between (20-120) neutrophils that are stained and need to be individually circled, thereafter by individually clicking in each circled cell and intensity reading for both ROCK (stained green) and PI3K (stained Red) is given and needs to be recorded manually. After completing this task which can be quite tedious as it can take up to 4 hours per 8 well dish. However when calculating co-localization coefficients of each marker, the ZEN lite program can generate a table for all the cells in the entire image, this can then be saved. Co-localization is defined as the presence of two or more different pixels occupying the same space, and in this case in the neutrophil. The value range

is between (0-1), 0 – no colocalization and 1 being all pixels are co-localized. The value is determined by the relative number of pixels in a channel (PI3K or ROCK) compared to the total number of pixels above the threshold.

3.5.8 Statistical Analysis

All statistical analysis was performed by Dr Martin Kidd, a trained and experienced biostatistician, at Stellenbosch University using Statistica (version 13.2). Data was analysed for normalcy of distribution, where after main effects of treatment was assessed by parametric and non-parametric ANOVA and Post Hoc Fischer's LSD tests. All data obtained from this analysis was then further analysed in Excel 2010. All Graphs data is presented at means and ± SEM (standard error of means), while tables are presented as means and ± SD (standard deviations). A p-value of <0.05 was considered statistically significant.

4. CHAPTER 4: RESULTS

4.1 Effects of Placebo and PCO Treatment on Haematological Parameters

Given the reports on altered adhesion molecule expression after PCO use, as well as anecdotal concerns about potential bleeding, full blood counts and clotting profiles were performed after the first 7 days of supplementation. Values for all participants were within expected normal ranges, with no evident effect of PCO as shown in **Table 4.1.1** and **Table 4.1.2.**

Table 4.1.1: Effects of placebo and PCO treatments on full blood count. *Blood sample analysis was performed on Day 7 for each participant. Data are expressed as means and standard deviations (SD). PCO - proanthocyanidolic oligomers, MCV - Mean Corpuscular Volume, MCH - Mean Cell Haemoglobin, MCHC - Mean Corpuscular Haemoglobin Concentration, RDW - Red cell Distribution Width. Statistical analysis: Levene's test for homogeneity of variance with Games-Howell post hoc test (ANOVA). Placebo n=9 and PCO n=9.*

		Placebo	**PCO**
Parameter	**Reference range**	**Mean (SD)**	**Mean (SD)**
Total leucocyte count	4.00 – 11.00 x 10^9/L	6.89 (1.83)	7.43 (0.90)
Neutrophils	2.00 – 7.50 x 10^9/L	3.83 (1.46)	4.14 (0.84)
Lymphocytes	1.00 – 4.00 x 10^9/L	2.56 (0.72)	2.86 (0.38)
Monocytes	0.00 – 0.80 x 10^9/L	0.30 (0.15)	0.23 (0.14)
Eosinophils	0.00 – 0.40 x 10^9/L	0.18 (0.10)	0.21 (0.05)
Fibrinogen	2.20 – 5.00 g/L	3.05 (0.50)	3.00 (0.41)
Platelet count	140 – 420 x 10^9/L	251 (63.26)	286.33 (41.65)
Total red cell count	3.70 – 5.30 x 10^{12}/L	4.92 (0.48)	4.83 (0.48)
Haemoglobin	11.50 – 15.50 g/dL	13.74 (1.22)	13.89 (1.19)
Haematocrit	0.35 – 0.45 L/L	0.42 (0.03)	0.42 (0.03)
MCV	81 – 96 fl	85.44 (3.97)	87.44 (7.45)
MCH	28 – 35pg	28.11 (1.62)	28.78 (3.03)
MCHC	32 – 37 g/dL	32.78 (0.44)	32.89 (0.78)
RDW	10 – 15%	13.30 (1.04)	12.87 (1.43)

Table 4.1.2: Effects of placebo and PCO treatments on clotting profile. *Blood sample analysis was performed on Day 7 for each participant. Data are expressed as means and standard deviations (SD). PT - Prothrombin time, INR - International Normalized Ratio, aPTT - activated clotting time, PCO - proanthocyanidolic oligomers. Statistical analysis: Levene's test for homogeneity of variance with Games-Howell post hoc test (ANOVA). Placebo n=9 and PCO n=9*

		PLACEBO	**PCO**
Parameter	**Reference range**	**Mean (SD)**	**Mean (SD)**
PT	11- 13.5 sec	12.54 (1.65)	11.68 (1.19)
INR	0.9 – 1.3	1.09 (0.11)	1.03 (0.05)
aPTT	25.4 – 38.4 sec	30.10 (2.83)	30.36 (3.18)

4.2 Effects of PCO Treatment on Neutrophil Migration

The assay used for neutrophil chemokinesis is novel. Representative images are presented for the placebo- (**Figure 4.2.1**) and PCO-treated groups (**Figure 4.2.2.).** In both figures, frames **A** (D0) and **C** (D14) shows the minimal movement of cells in absence of a chemotactic signal, while frames **B** and **D** indicate substantially more movement when exposed to an fMLP signal. No difference was observed over time or between treatment groups.

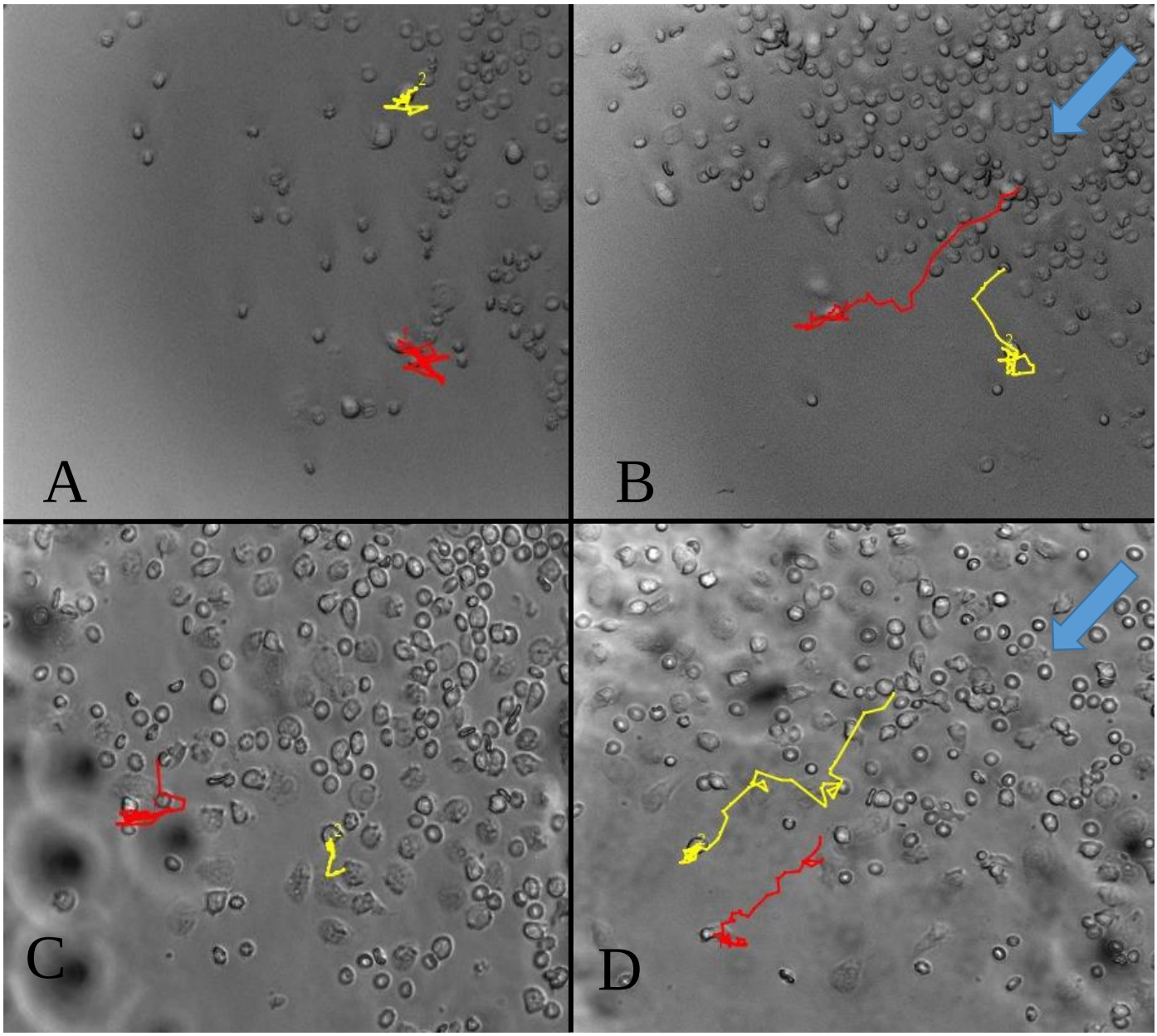


Figure 4.2.1 Representative images of placebo (untreated) neutrophil chemokinesis showing migrational movement of unstimulated neutrophils on day 0 (A) and day 14 (C), as well as fMLP-stimulated neutrophils at the same time points (B and D). *The migrational pathway tracked for the neutrophils is seen by the coloured lines on each image. The blue arrows indicate the direction the neutrophils should move towards the chemoattractant added. PCO - proanthocyanidolic oligomers, fMLP - N-Formylmethionine-leucyl-phenylalanine. Images taken at 20x magnification. Placebo D0 unstimulated n=6, Placebo D0 stimulated n=6, Placebo D14 unstimulated n=9, Placebo D14 stimulated n=9*

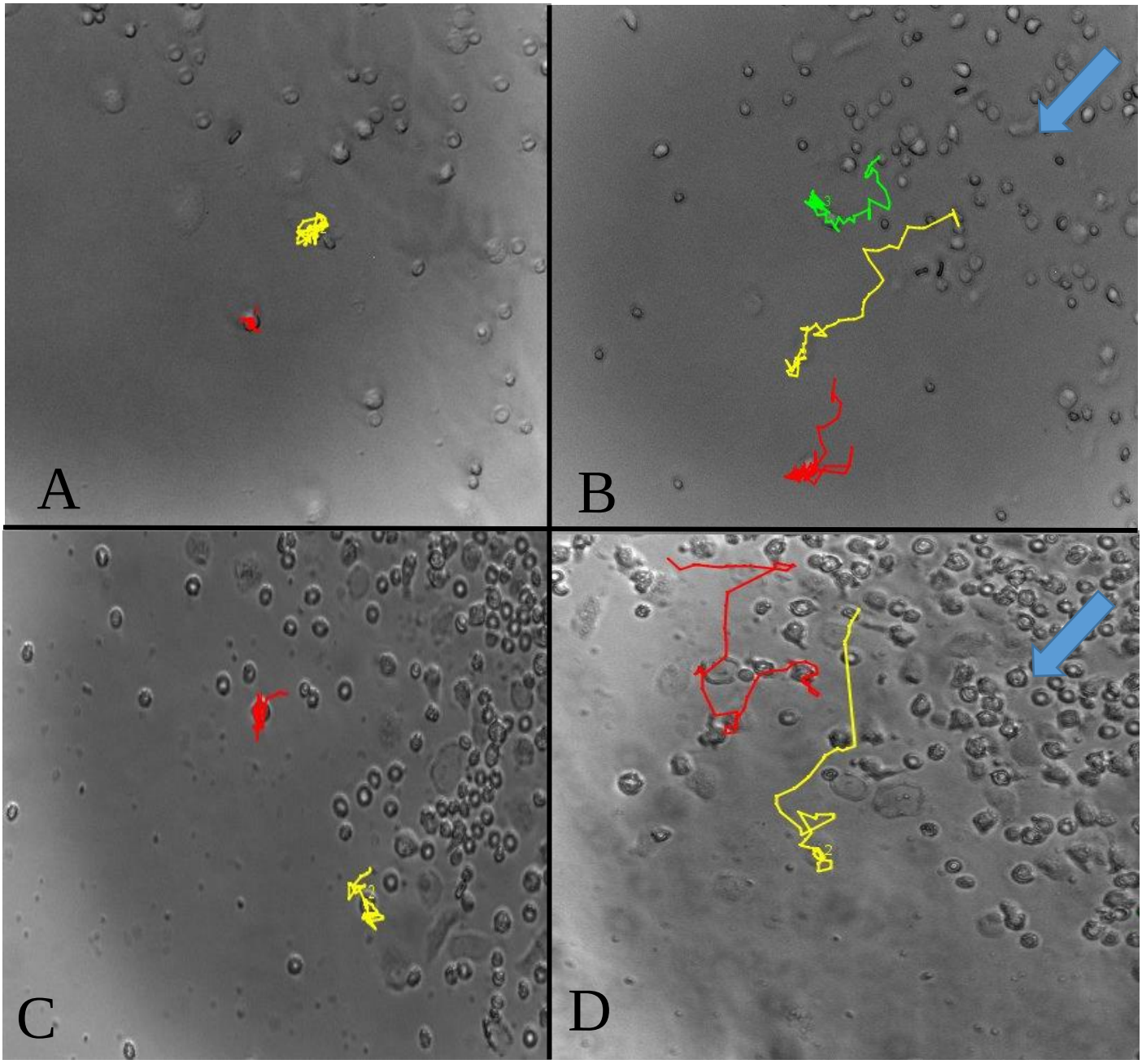


Figure 4.2.2 Representative images of PCO treated neutrophil chemokinesis showing migrational movement of unstimulated neutrophils on day 0 (A) and day 14 (C), as well as fMLP-stimulated neutrophils at the same time points (B and D). *The migrational pathway tracked for the neutrophils is seen by the coloured lines on each image. The blue arrows indicate the direction the neutrophils should move towards the chemoattractant added. PCO - proanthocyanidolic oligomers, fMLP - N-Formylmethionine-leucyl-phenylalanine. Images taken as 20x magnification. PCO D0 unstimulated n=6, PCO D0 stimulated n=6, PCO D14 unstimulated n=9, PCO D14 stimulated n=9*

Numerical data obtained by performing analysis of variance (ANOVA) test on the effects of chemotaxin stimulation on total and linear distance travelled by neutrophils are presented in **Figures 4.2.3** and **4.2.4**. Findings from this analysis confirm the qualitative results shown earlier (**Figures 4.2.1 and 4.2.2**) and hence the validity of the model. Data indicates that fMLP stimulation significantly increased the total (≈60%; ANOVA main effect of stimulation, $P < 0.05$) and linear (≈185%; ANOVA main effect of stimulation, $P < 0.05$) neutrophil distance travelled compared to unstimulated total and linear neutrophil distance travelled. Group-specific quantitative results confirmed visual results, with no significant treatment or time effect (**Table 4.2**).

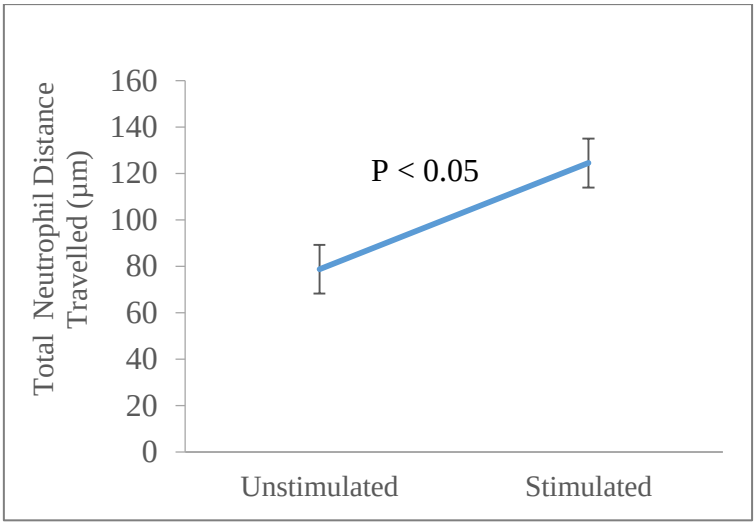


Figure 4.2.3 Effect of fMLP stimulation on total distance travelled by neutrophils. *Data are expressed as means and standard error of means (SEM). Statistical analysis: Post Hoc Fischer's LSD test (ANOVA). fMLP - N-Formylmethionine-leucyl-phenylalanine. Unstimulated n=30, Simulated n=30.*

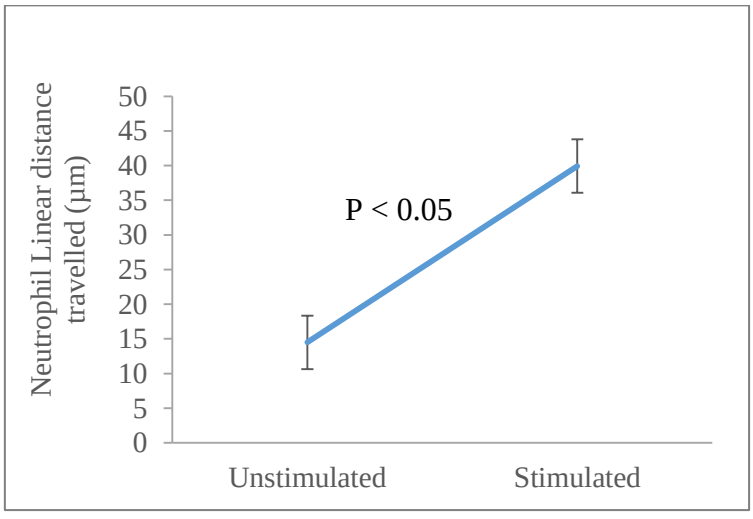


Figure 4.2.4 Effect of fMLP stimulation on linear distance travelled by neutrophils. *Data are expressed as means and standard error of means. Statistical analysis: Post Hoc Fischer's LSD test (ANOVA). fMLP - N-Formylmethionine-leucyl-phenylalanine. Unstimulated n=30, Simulated n=30.*

Table 4.2 The distance travelled by neutrophil on day 0 (D0) and day 14 (D14) for placebo and PCO treated groups under stimulated and unstimulated conditions. *Values are expressed as means and standard deviations (SD). PCO- proanthocyanidolic oligomers, fMLP- N-Formylmethionine-leucyl-phenylalanine. Statistical analysis: Post Hoc Fischer's LSD test (ANOVA). Placebo D0 unstimulated n=6, Placebo D0 stimulated n=6, Placebo D14 unstimulated n=9, Placebo D14 stimulated n=9. PCO D0 unstimulated n=6, PCO D0 stimulated n=6, PCO D14 unstimulated n=9, PCO D14 stimulated n=9*

Neutrophil chemokinetic indicators	**PLACEBO**				**PCO**			
	Unstimulated		**Stimulated**		**Unstimulated**		**Stimulated**	
	D0	**D14**	**D0**	**D14**	**D0**	**D14**	**D0**	**D14**
	Mean (SD)	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**
Total Distance (um)	65.08 (37.30)	73.26 (35.46)	83.32 (54.66)	143.21 (60.77)	92.64 (56.14)	81.24 (43.68)	112.90 (69.78)	155.78 (67.11)
Linear distance (um)	12.67 (13.93)	16.61 (10.470	26.66 (16.57)	53.19 (29.16)	13.54 (10.71)	13.52 (42.91)	35.30 (25.27)	42.91 (29.01)

4.3 Effects of PCO Treatment on Expression of Selected molecular Proteins in Neutrophils

The result obtained for CD66b expression in neutrophils is presented in **Figure 4.3.1**. There was no significant difference between treatment groups. The expression of CD66b at day 0 was not significantly different from that observed at day 14 for both placebo and PCO-treated groups, although the average expression was lower for both groups on day 14. Similar effects were observed for the expression of adhesion markers ICAM and VCAM in neutrophils as shown in **Figure 4.3.2 and 4.3.3** respectively. No significant difference was observed between treatments even after 14 days interval. The representative flow cytometric panels of unstained and multiple stained neutrophil population against CD66b, ICAM and VCAM antibodies are shown in **Figures 4.3.4 and 4.3.5** which indicate that the markers used are expressed and indeed cross the threshold.

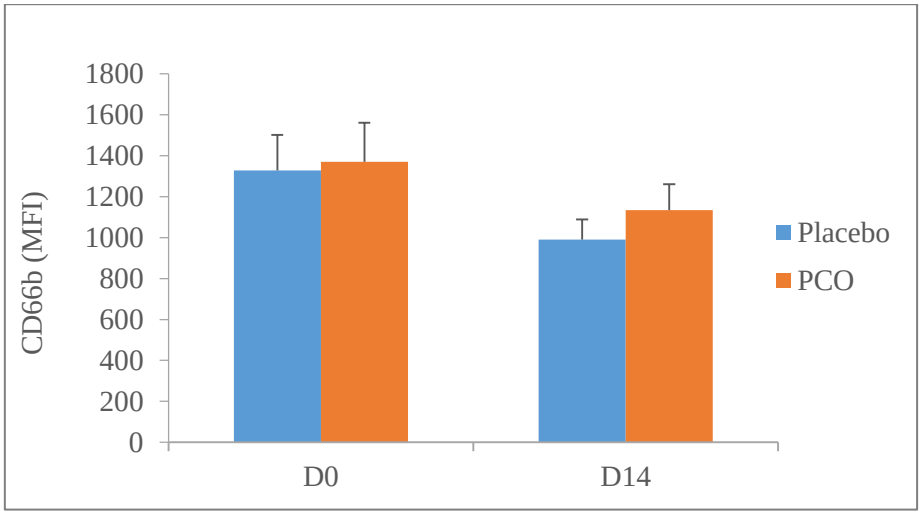


Figure 4.3.1 Effects of placebo and PCO treatments on expression of CD66b in neutrophils at day 0 (D0) and day 14 (D14) of supplementation. *Data are expressed as means and standard error of means. MFI - median fluorescent intensity in arbitrary units; PCO - proanthocyanidolic oligomers, MFI- Median Fluorescent Intensity. Statistical analysis: Post Hoc Fischer's LSD test (ANOVA). Placebo D0 n=9, PCO D0 n=9, Placebo D14 n=9, PCO D14 n=9.*

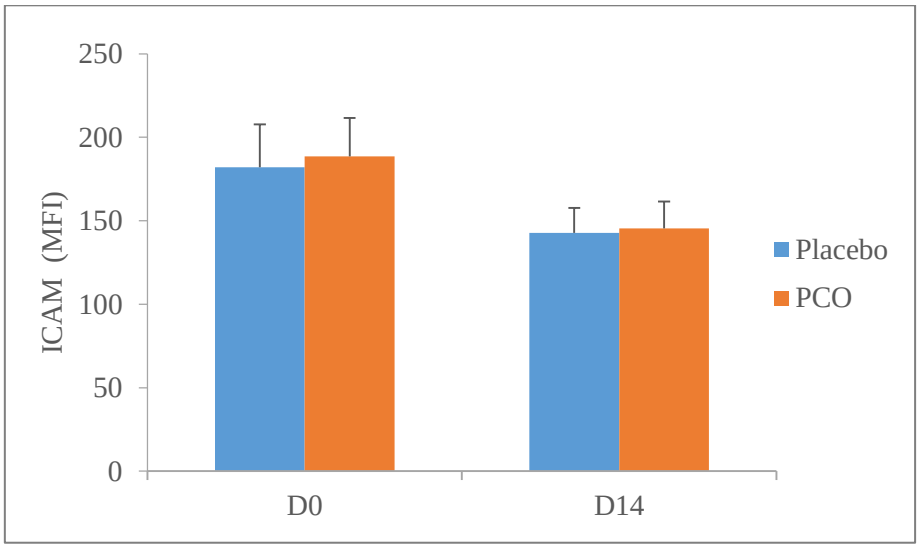


Figure 4.3.2 Effects of placebo and PCO treatments on expression of ICAM in neutrophils at day 0 (D0) and day 14 (D14) of supplementation. *Data are expressed as means and standard errors of means. PCO - proanthocyanidolic oligomers, MFI - median fluorescent intensity in arbitrary units, ICAM – Intracellular Adhesion Molecule. Statistical analysis: Post Hoc Fischer's LSD test (ANOVA). Placebo D0 n=9, PCO D0 n=9, Placebo D14 n=9, PCO D14 n=9.*

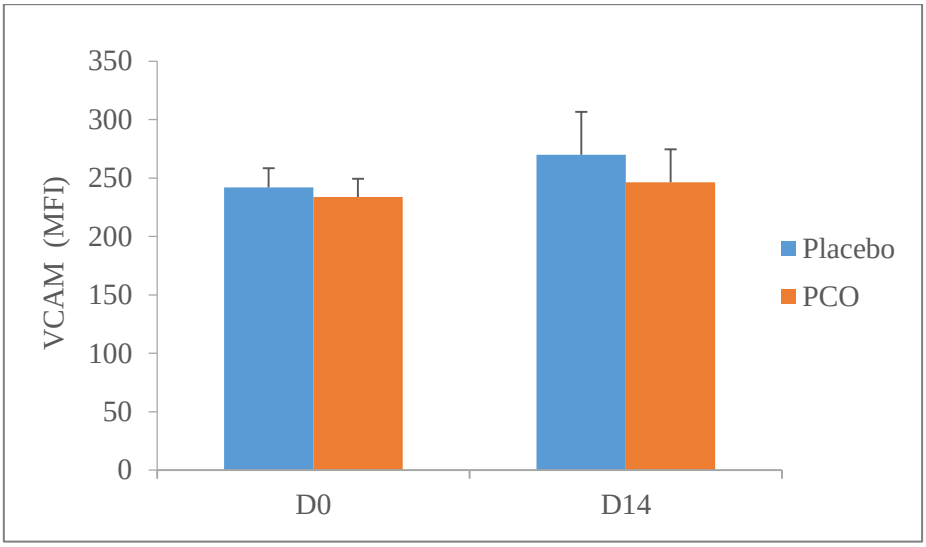
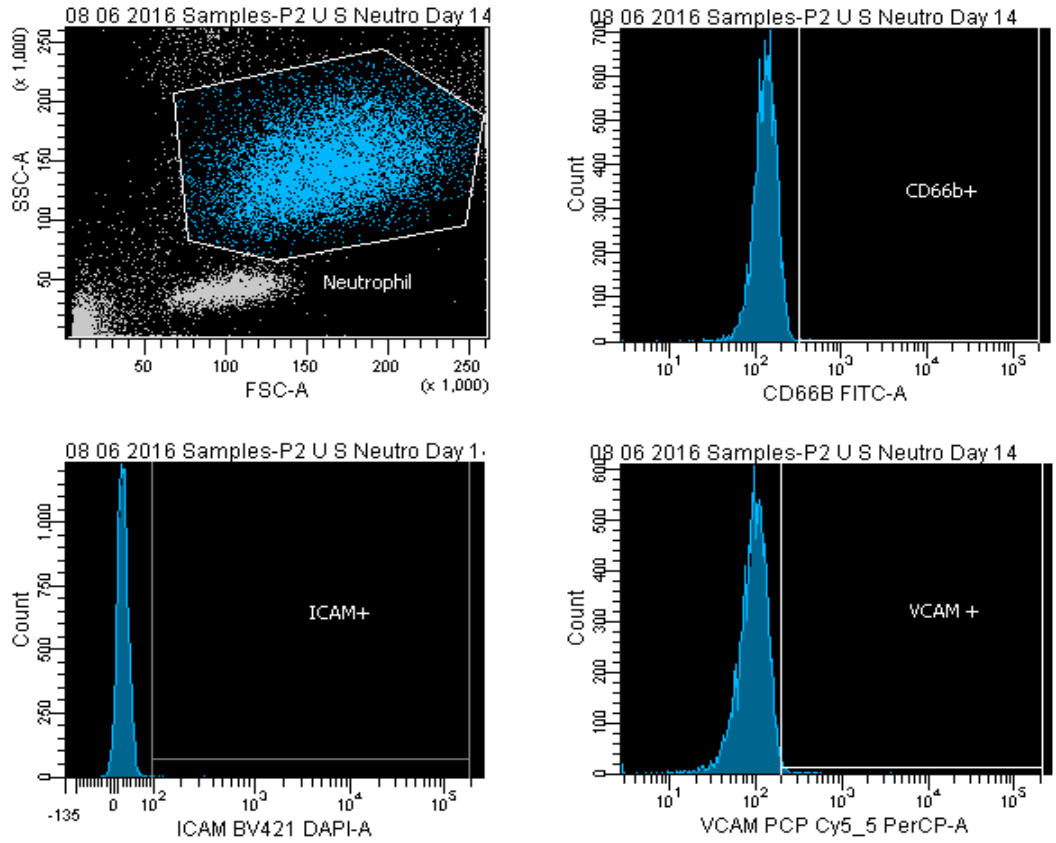


Figure 4.3.3 Effects of placebo and PCO treatments on expression of VCAM in neutrophils at day 0 (D0) and day 14 (D14) of supplementation. *Data are expressed as means and standard errors of means. MFI - median fluorescent intensity in arbitrary units, PCO - proanthocyanidolic oligomers, VCAM – Vascular Adhesion Molecule. Statistical analysis: Post Hoc Fischer's LSD test (ANOVA). Placebo D0 n=9, PCO D0 n=9, Placebo D14 n=9, PCO D14 n=9.*



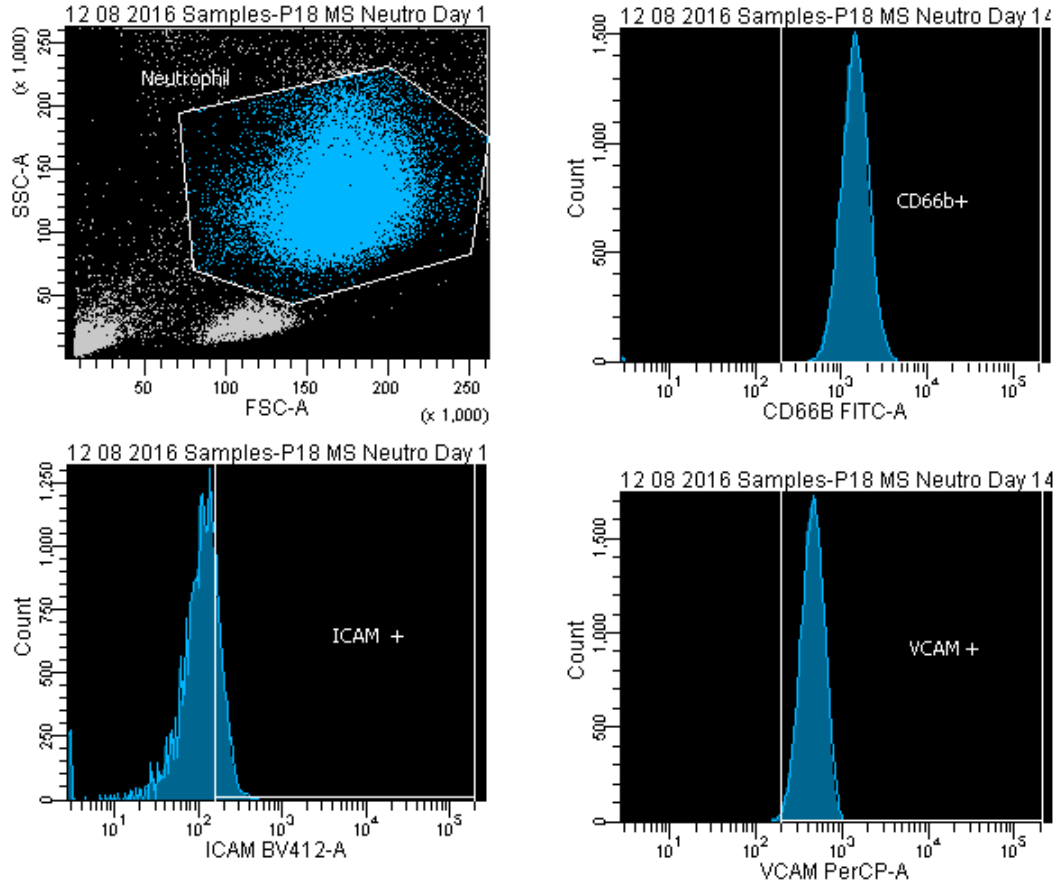
Tube: P2 U S Neutro Day 14

Population	#Events	%Parent	%Total
All Events	15,361	####	100.0
Neutrophil	10,126	65.9	65.9
CD66b+	23	0.2	0.1
VCAM +	97	1.0	0.6
ICAM+	3	0.0	0.0

Experiment Name: Neutrophils_ICAM_VCAM
Specimen Name: 08 06 2016 Samples
Tube Name: P2 U S Neutro Day 14

Population	Parent Name	#Events	%Parent	CD66B FIT... Geo Mean	VCAM PCP ... Geo Mean	ICAM BV421... Geo Mean
Neutrophil	All Events	10,126	65.9	####	####	####
CD66b+	Neutrophil	23	0.2	565	401	42
VCAM +	Neutrophil	97	1.0	274	247	####
ICAM+	Neutrophil	3	0.0	1,909	651	168

Figure 4.3.4 Representative flow cytometric panels showing unstained neutrophil population stained against CD66b, ICAM and VCAM antibodies. *Vertical lines indicate fluorescence thresholds while unstained populations found on the left side of the threshold are indicative of negative staining for the fluorescent labels. Placebo D0 n=9, PCO D0 n=9, Placebo D14 n=9, PCO D14 n=9.*



Tube: P18 MS Neutro Day 14

Population	#Events	%Parent	%Total
All Events	43,707	####	100.0
Neutrophil	30,000	68.6	68.6
CD66b+	29,690	99.0	67.9
VCAM +	29,647	98.8	67.8
ICAM +	5,392	18.0	12.3

Experiment Name: Neutrophils_ICAM_VCAM_Aug 2016
Specimen Name: 12 08 2016 Samples
Tube Name: P18 MS Neutro Day 14

Population	#Events	%Parent	CD66B FITC-A Geo Mean	VCAM PerCP-A Geo Mean	ICAM BV412-A Geo Mean
Neutrophil	30,000	68.6	####	####	####
CD66b+	29,690	99.0	1,342	####	####
VCAM +	29,647	98.8	####	423	####
ICAM +	5,392	18.0	####	508	198

Figure 4.3.5 Representative flow cytometric panels showing multiple stained neutrophil population stained against CD66b, ICAM and VCAM fluorescent antibodies. *Vertical lines indicate fluorescence thresholds while multiple stained populations found on the right side of the thresholds are indicative of positive staining for the fluorescent labels. Placebo D0 n=9, PCO D0 n=9, Placebo D14 n=9, PCO D14 n=9.*

4.4 Effects of PCO Treatment on Neutrophil Migrational Mechanisms

Representative images obtained from the expression of intracellular indicators of neutrophil polarisation, ROCK and PI3K, when neutrophils are supplemented with placebo and PCO are shown in **Figure 4.4.1** and **Figure 4.4.2** respectively. From the qualitative data, it seems that more co-localization occurs under stimulated conditions. Pixels seem more dispersed in images A and C (unstimulated), when compared to B and D (stimulated).

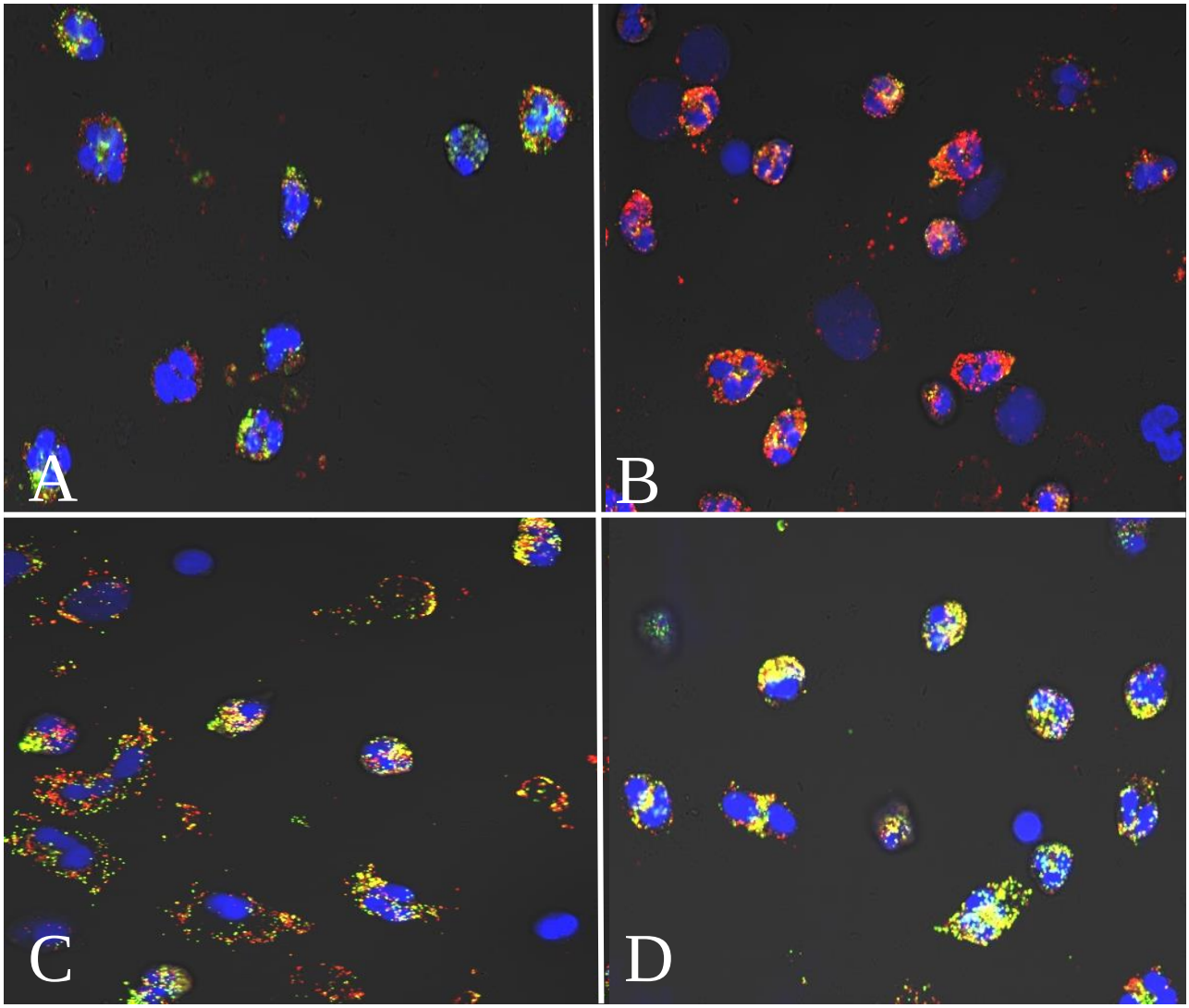


Figure 4.4.1: Representative images of fixed and stained neutrophils from placebo-supplemented individuals after migration. *(A) Stained neutrophils on Day 0 that migrated towards RPMI (unstimulated); (B) Stained neutrophils on Day 0 that migrated towards the fMLP chemoattractant (stimulated); (C) Stained neutrophils on Day 14 that migrated towards RPMI (unstimulated); (D) Stained neutrophils on Day 14 that migrated towards the fMLP chemoattractant (stimulated). ROCK stained green colour while PI3K stained red. The two colours seemed to co-localize which is why the yellow colour can be seen. Dappi (1:20 dilution) was used and stained the neutrophil nuclei blue. fMLP - N-Formylmethionine-leucyl-phenylalanine, PI3K – Phosphoinositide 3-Kinase, ROCK- Rho-associated coil-containing protein kinase . Images taken at 20x magnification. Placebo D0 n=8, PCO D0 n=8, Placebo D14 n=9, PCO D14 n=9.*

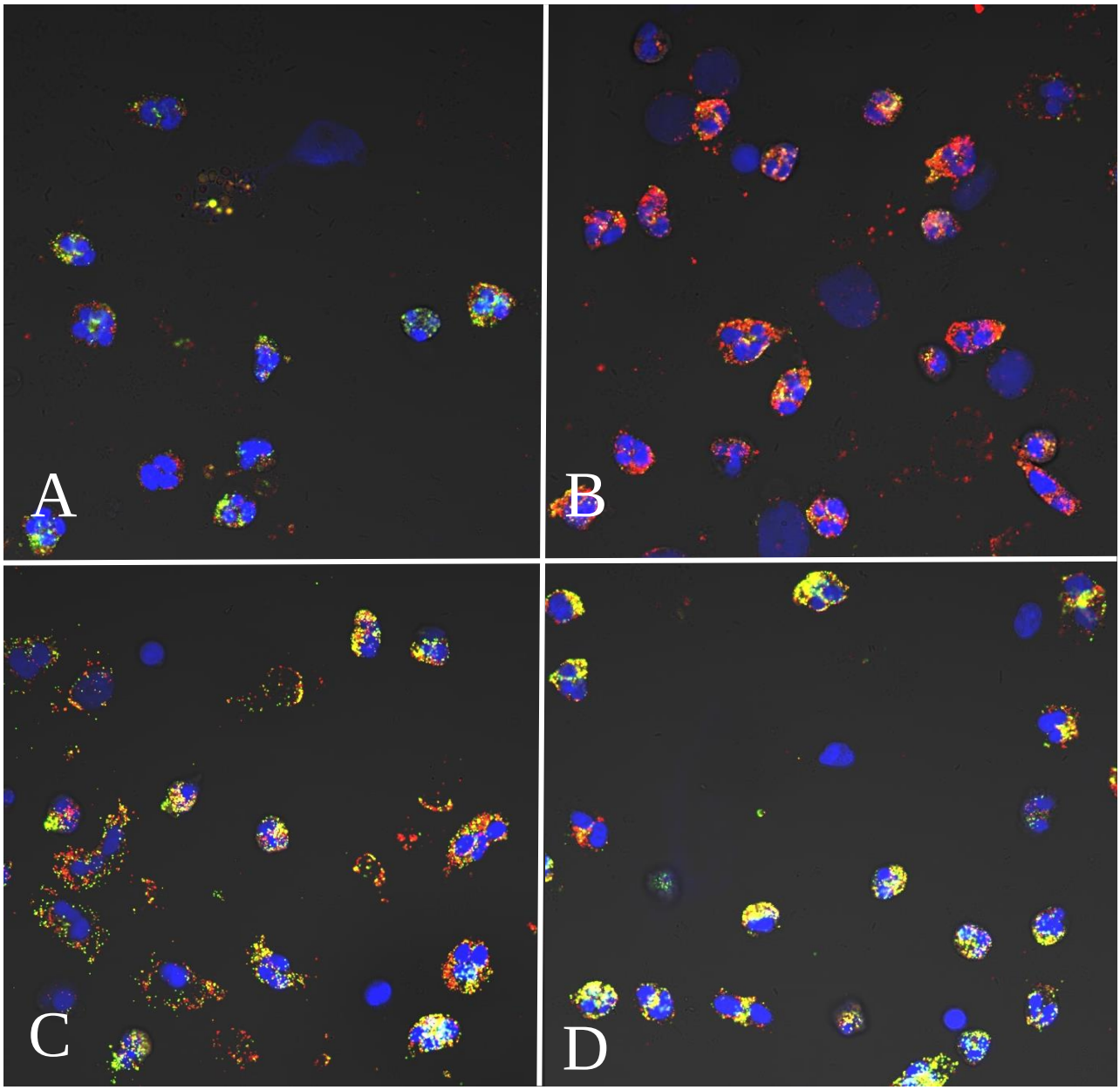


Figure 4.4.2: Representative images of fixed and stained neutrophils from PCO-supplemented individuals after migration. *(A) Stained neutrophils on Day 0 that migrated towards RPMI (unstimulated); (B) Stained neutrophils on Day 0 that migrated towards the fMLP chemoattractant (stimulated); (C) Stained neutrophils on Day 14 that migrated towards RPMI (unstimulated); (D) Stained neutrophils on Day 14 that migrated towards the fMLP chemoattractant (stimulated). ROCK stained green colour while PI3K stained red. The two colours seemed to co-localize which is why the yellow colour can be seen. Dappi (1:20 dilution) was used and stained the neutrophil nuclei blue. PCO - proanthocyanidolic oligomers, fMLP - N-Formylmethionine-leucyl-phenylalanine, PI3K – Phosphoinositide 3-Kinase, ROCK- Rho-associated coil-containing protein kinase . Images taken at 20x magnification. Placebo D0 n=8, PCO D0 n=8, Placebo D14 n=9, PCO D14 n=9.*

Upon quantification of the images shown above, both ROCK and PI3K expression were highly variable between individuals, as well as over time (**Figures 4.4.3 and 4.4.4.).** In addition, no statistical significant effect on these parameters was evident with PCO treatment although there seems to be a deviation in ROCK expression after PCO treatment (PCO day 14 different from all other conditions). Also, there exists a tendency for an interaction effect

(P=0.06) of treatment, time and fMLP stimulation (**Figure 4.4.3**). From the graph, it is possible that this effect is due to a different response to fMLP stimulation after 14 days of PCO treatment, as the ROCK expression decreased by more than 50% after stimulation.

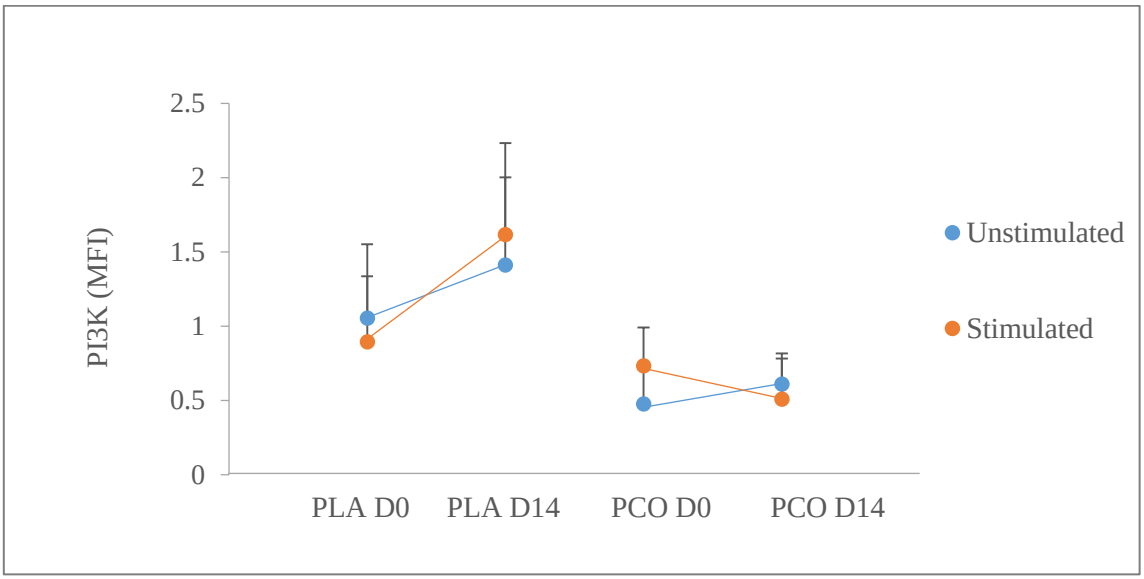


Figure 4.4.3 Expression of PI3K in neutrophils from placebo and active groups under unstimulated and stimulated conditions at day 0 (D0) and day 14 (D14). *Data are expressed as means and standard errors of means (SEM). PCO - proanthocyanidolic oligomers. PI3K - Phosphoinositide 3-Kinase, MFI-median Fluorescent Intensity. Statistical analysis: Post Hoc Fischer's LSD test (ANOVA). Placebo D0 n=8, PCO D0 n=8, Placebo D14 n=9, PCO D14 n=9.*

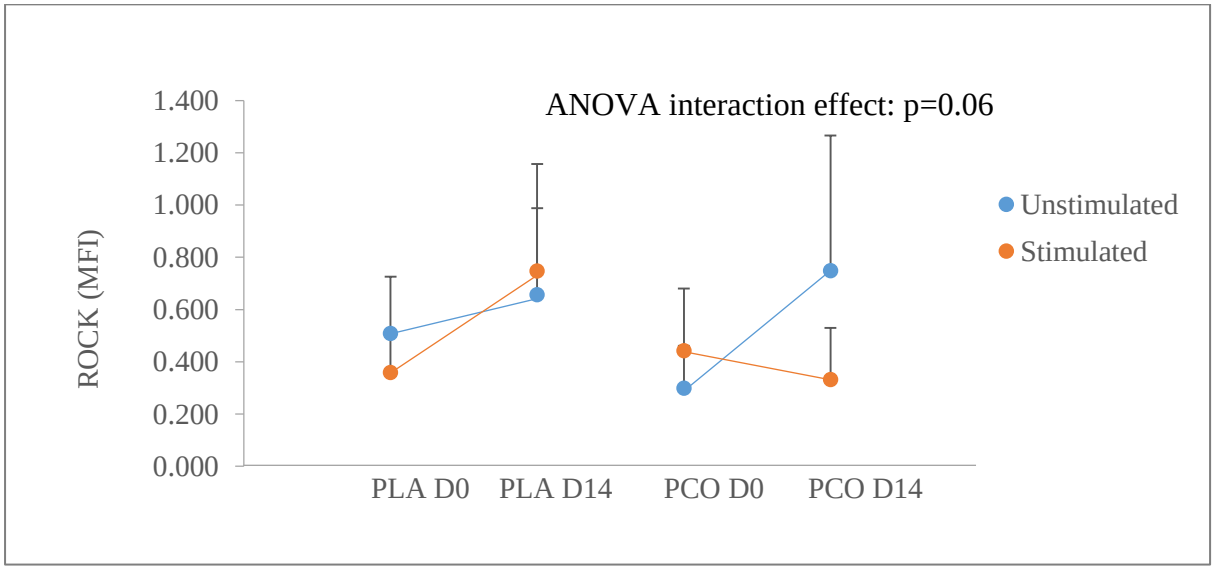


Figure 4.4.4 Expression of ROCK in neutrophils from placebo and active groups under unstimulated and stimulated conditions at day 0 (D0) and day 14 (D14). *Data are expressed as means and standard errors of mean (SEM). PCO - proanthocyanidolic oligomers. ROCK - Rho-associated coil-containing protein kinase, MFI-median Fluorescent Intensity. Statistical analysis: Post Hoc Fischer's LSD test (ANOVA). Placebo D0 n=8, PCO D0 n=8, Placebo D14 n=9, PCO D14 n=9.*

Furthermore, an aberration in ROCK co-localisation with PI3K was seen in neutrophils treated with PCO when compared to that of placebo group as shown in **Figure 4.4.5**. Also, fMLP stimulation significantly (P<0.05) increased the relative portion of ROCK co-localisation with PI3K as a result of treatments, once again validating the novel model. For both D0 groups, as well as D14 PLA, ROCK co-localisation increased by more than 25% (although not statistically significantly; (**Figure 4.4.6**), while in contrast, ROCK co-localisation in the PCO-treated group on D14 did not change on stimulation. Taken together, these data suggest a relatively greater sensitivity to modulation by PCO for ROCK expression compared to PI3K, where no differences in total expression or co-localisation was observed (**Figure 4.4.7)**.

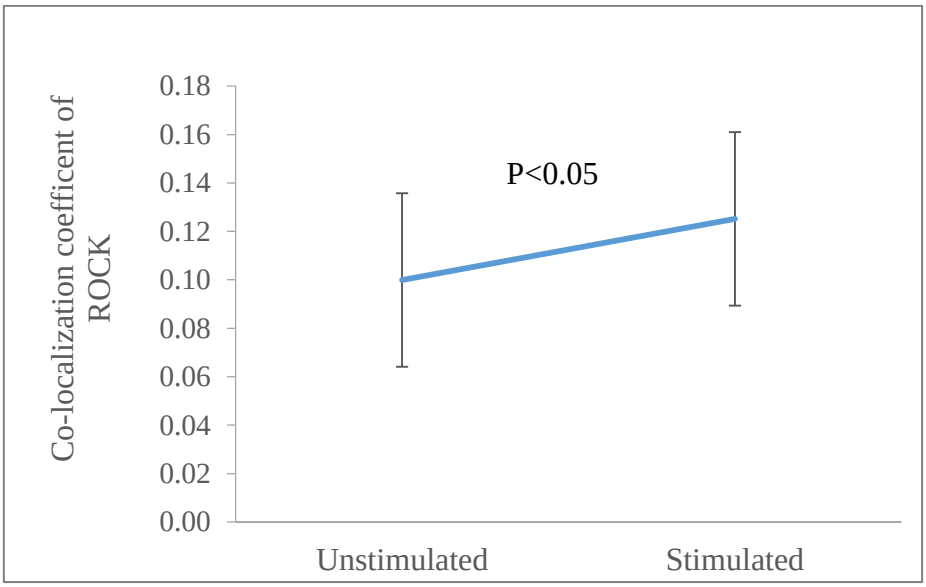


Figure 4.4.5 Effects of fMLP stimulation on co-localization coefficient of ROCK expression in neutrophils. *ANOVA main effect of treatment, P<0.05 Data are expressed as means and standard errors of mean (SEM). Statistical analysis: Post Hoc Fischer's LSD test (ANOVA) ROCK- Rho-associated coil-containing protein kinase, Co-localization coefficient – relative number of co-localizing pixels in ROCK respectively, as compared to the total number of pixels above the threshold (PI3K and ROCK combined). Unstimulated n= 34, Stimulated n=34.*

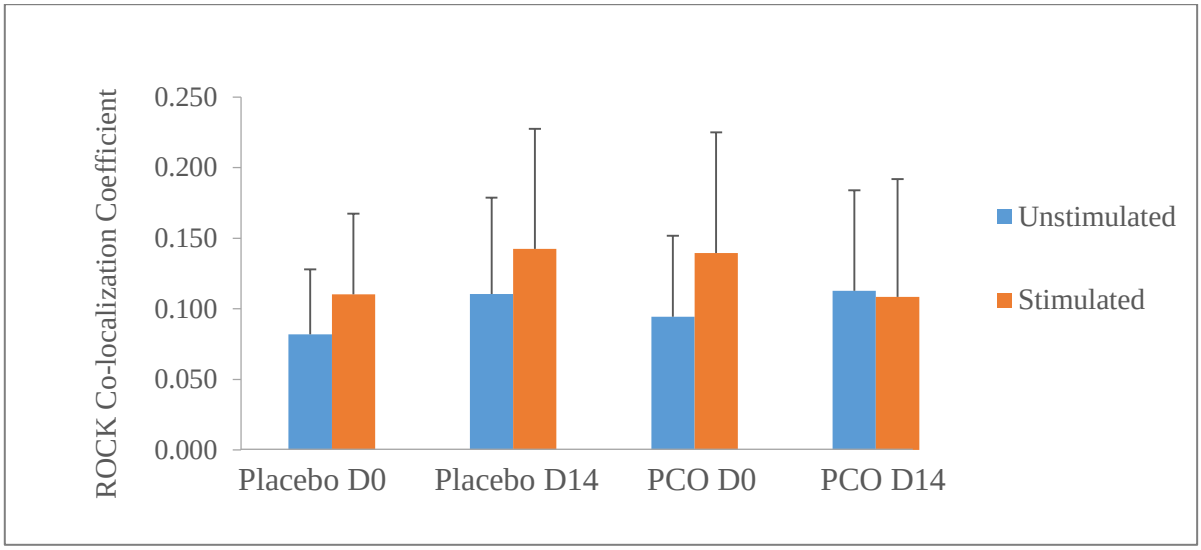


Figure 4.4.6 Co-localization coefficient of ROCK expression in neutrophils from placebo and active groups under unstimulated and stimulated conditions at day 0 (D0) and day 14 (D14). *Data are expressed as means and standard errors of mean (SEM). Statistical analysis: Post Hoc Fischer's LSD test (ANOVA). ROCK - Rho-associated coil-containing protein kinase, PCO - proanthocyanidolic oligomers, Co-localization coefficient – relative number of co-localizing pixels in ROCK respectively, as compared to the total number of pixels above the threshold (PI3K and ROCK combined PCO - proanthocyanidolic oligomers, ROCK - Rho-associated coil-containing protein kinase, PI3K - Phosphoinositide 3-Kinase. Placebo D0 n=8, PCO D0 n=8, Placebo D14 n=9, PCO D14 n=9.*

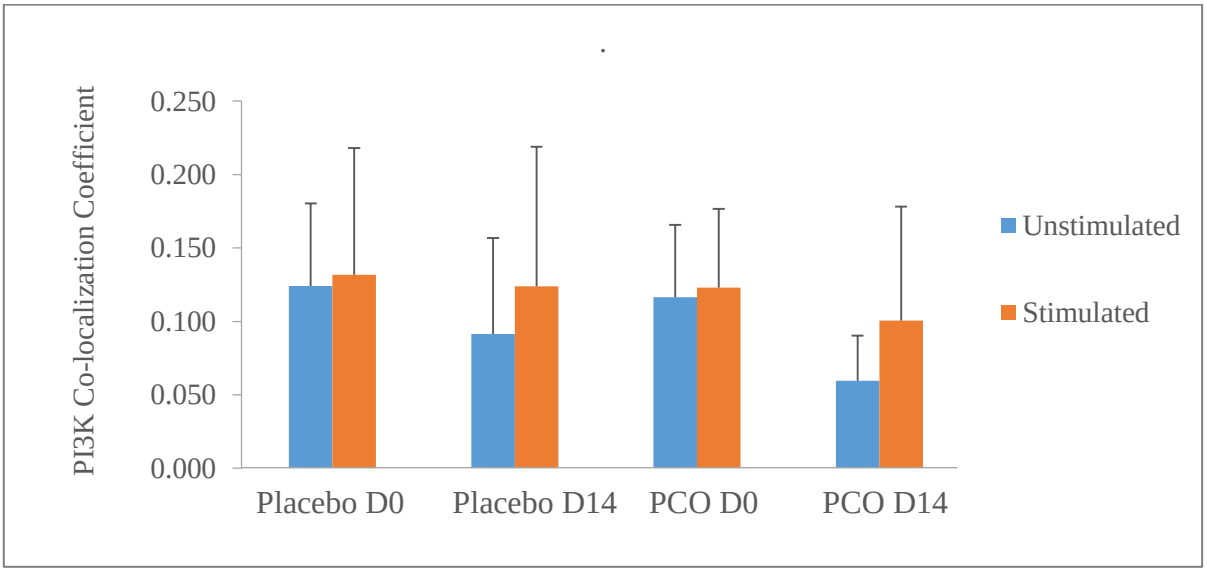


Figure 4.4.7 Co-localization coefficient of PI3K expression in neutrophils from placebo and active groups under unstimulated and stimulated conditions at day 0 (D0) and day 14 (D14). *Data are expressed as means and standard errors of mean (SEM). Statistical analysis: Post Hoc Fischer's LSD test (ANOVA). PI3K - Phosphoinositide 3-Kinase, PCO - proanthocyanidolic oligomers, ROCK - Rho-associated coil-containing protein kinase Co-localization coefficient- relative number of co-localizing pixels in PI3K respectively, as compared to the total number of pixels above the threshold (PI3K and ROCK combined). Placebo D0 n=8, PCO D0 n=8, Placebo D14 n=9, PCO D14 n=9.*

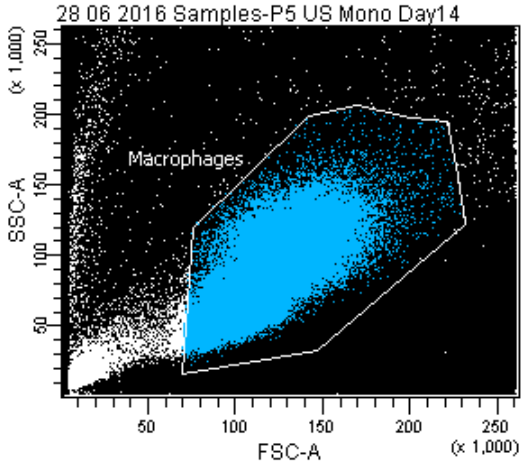
4.5 Effect of PCO Treatment on Macrophage Phenotype

The expression of macrophage phenotype markers in neutrophils from treatment groups over time are presented in **Table 4.5.** No main effect of treatment or over time was seen, although it should be noted that both intra- and inter-individual variations were quite large.

Table 4.5 Effects of placebo and PCO on macrophage markers in neutrophils at day 0 (D0) and day 14 (D14) after treatments*. Data are expressed as means and standard deviation (SD). MFI-Median Fluorescent Intensity PCO - proanthocyanidolic oligomers, MPO –Myeloperoxidase, HLA-DR – Human leukocyte antigen D, IL-10 Interleukin-10. Statistical analysis: LSD test (ANOVA). Placebo D0 n=9, PCO D0 n=9, Placebo D14 n=9, PCO D14 n=9.*

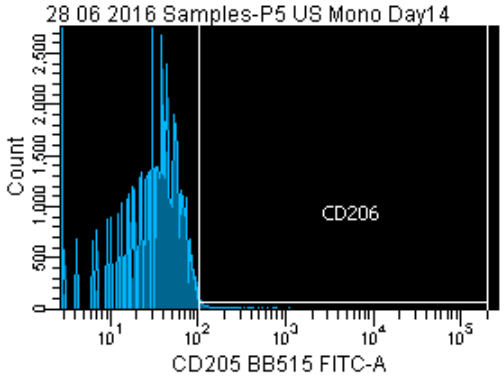
Macrophage Phenotypic Markers	**Placebo Do**	**Placebo D14**	**PCO D0**	**PCO D14**
	Mean (SD)	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**
CD86 MFI	1071.67 (493.78)	1344.89 (468.68)	1086.44 (436.61)	1284.226 (84.83)
HLA-DR MFI	1144.67 (602.05)	1513.33 (607.96)	827.33 (402.60)	1131.33 (691.58)
CD274 MFI	601.44 (370.66)	669.67 (405.02)	557.56 (331.41)	450.33 (198.01)
MPO MFI	1940.44 (1426.93)	1819.00 (1813.36)	1838.67 (1020.85)	1868.89 (1658.63)
CD206 MFI	1895.56 (1620.96)	2027.22 (1204.05)	1474.11 (1408.42)	1440.44 (953.22)
CD163 MFI	464.56 (186.56)	543.11 (186.99)	487.33 (219.20)	488.11 (194.85)
IL-10 MFI	256.67 (170.83)	195.67 (66.09)	178.00 (46.78)	199.44 (97.42)

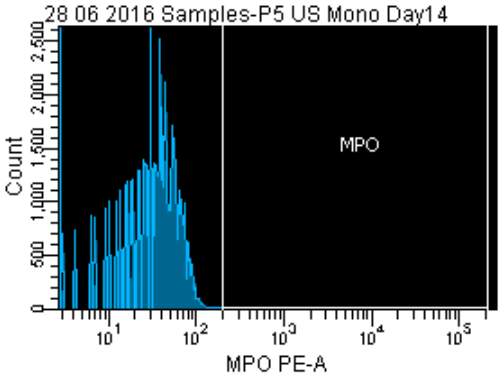
Figures 4.5.1 and **4.5.2** illustrate representative flow cytometric panels showing unstained and multiple stained macrophage population. The data indicates that the markers used are thus expressed and indeed cross the threshold.

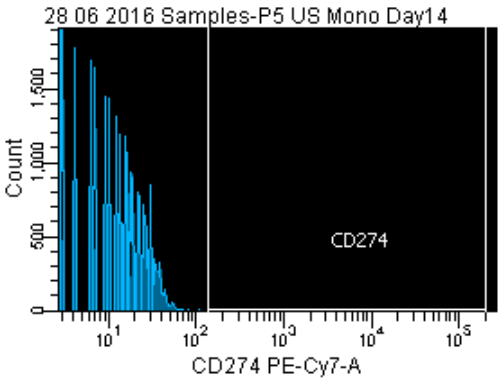


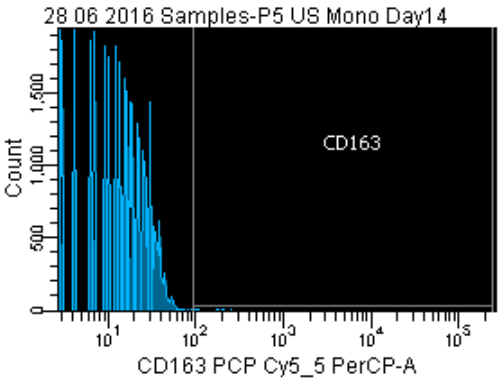
Tube: P5 US Mono Day14

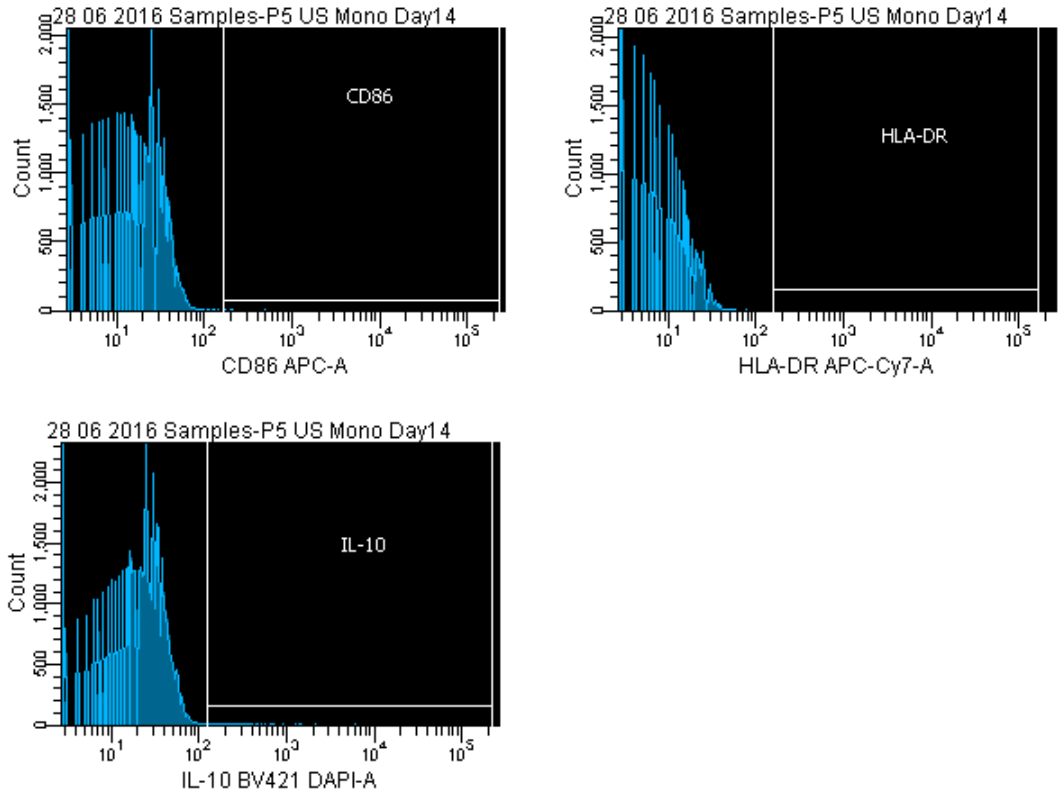
Population	#Events	%Parent	%Total
All Events	65,855	####	100.0
Macrophages	53,121	80.7	80.7
CD206	424	0.8	0.6
CD163	13	0.0	0.0
CD274	1	0.0	0.0
MPO	43	0.1	0.1
CD86	5	0.0	0.0
HLA-DR	0	0.0	0.0
IL-10	130	0.2	0.2







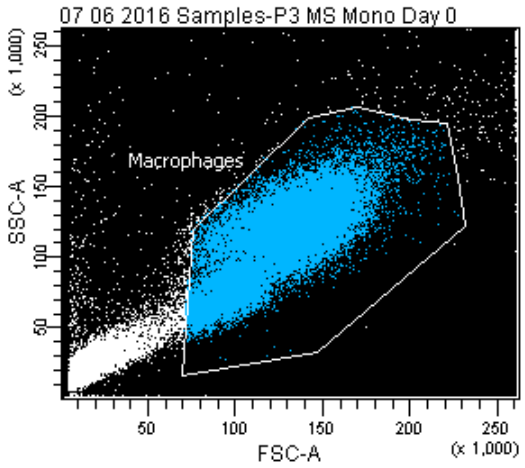




Experiment Name: Macrophage Typing_May 2016_2
Specimen Name: 28 06 2016 Samples
Tube Name: P5 US Mono Day14

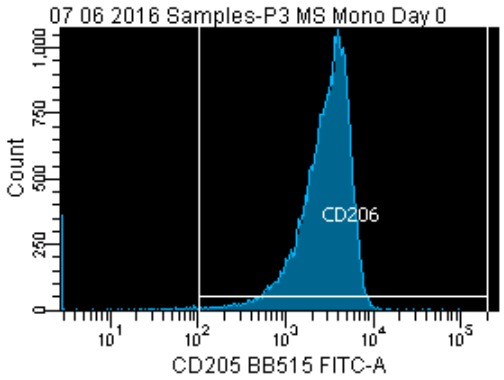
Population	#Events	%Parent	CD205 BB... Median	MPO PE-A Median	CD163 PC... Median	CD274 PE... Median	CD86 AP... Median	HLA-DR ... Median	IL-10 BV4... Median
Macrophages	53,121	80.7	34	32	6	-2	12	0	20
CD206	424	0.8	115	90	29	17	33	8	59
CD163	13	0.0	269	332	179	60	133	25	278
CD274	1	0.0	236	266	199	153	133	53	113
MPO	43	0.1	246	266	64	9	65	10	261
CD86	5	0.0	485	623	179	55	198	27	1,420
HLA-DR	0	0.0	####	####	####	####	####	####	####
IL-10	130	0.2	135	123	24	2	31	6	192

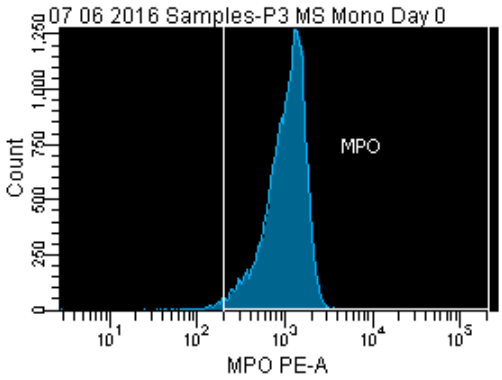
Figure 4.5.1 Representative flow cytometric panels showing unstained macrophage population stained against M1 (CD86, HLA-DR, CD274, MPO) and M2 (CD206, CD163, IL-10) fluorescent antibodies. *Vertical lines indicate fluorescence thresholds while unstained populations found on the left side of the thresholds are indicative of negative staining for the fluorescent labels. Placebo D0 n=9, PCO D0 n=9, Placebo D14 n=9, PCO D14 n=9*

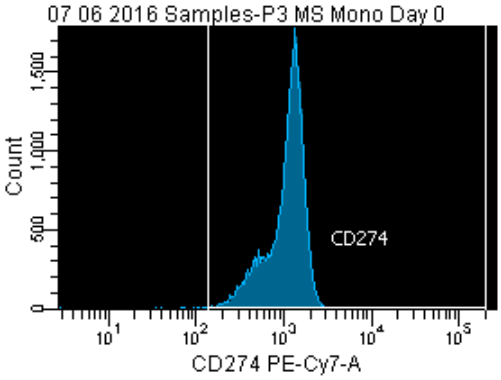


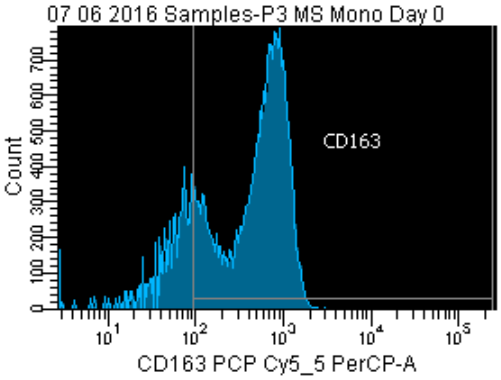
Tube: P3 MS Mono Day 0

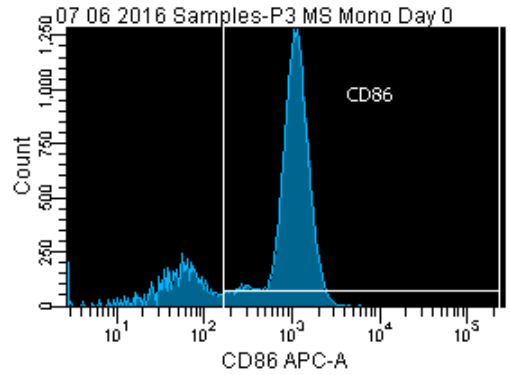
Population	#Events	%Parent	%Total
All Events	65,493	####	100.0
Macrophages	29,158	44.5	44.5
CD206	28,654	98.3	43.8
CD163	23,034	79.0	35.2
CD274	29,130	99.9	44.5
MPO	28,829	98.9	44.0
CD86	23,518	80.7	35.9
HLA-DR	28,427	97.5	43.4
IL-10	17,171	58.9	26.2

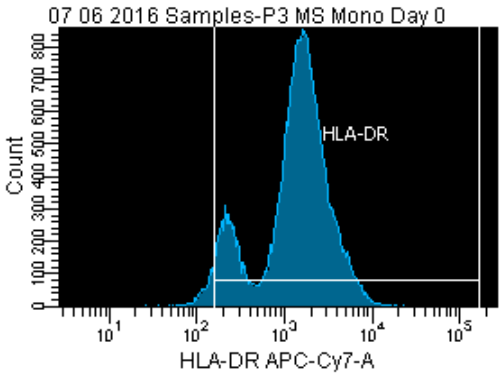


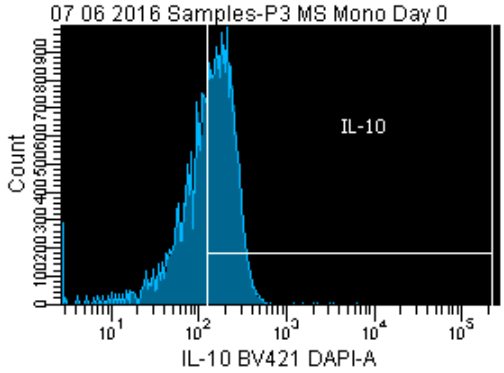












Experiment Name: Macrophage Typing_May 2016_2
Specimen Name: 07 06 2016 Samples
Tube Name: P3 MS Mono Day 0

Population	#Events	%Parent	CD205 BB... Median	MPO PE-A Median	CD163 PC... Median	CD274 PE... Median	CD86 AP... Median	HLA-DR ... Median	IL-10 BV4... Median
Macrophages	29,158	44.5	3,108	1,110	483	1,201	978	1,488	142
CD206	28,654	98.3	3,151	1,120	494	1,208	987	1,506	144
CD163	23,034	79.0	3,612	1,246	626	1,307	1,078	1,702	164
CD274	29,130	99.9	3,111	1,110	483	1,201	978	1,489	142
MPO	28,829	98.9	3,137	1,117	491	1,205	984	1,501	143
CD86	23,518	80.7	3,528	1,234	616	1,295	1,083	1,733	160
HLA-DR	28,427	97.5	3,169	1,128	500	1,214	992	1,518	144
IL-10	17,171	58.9	4,063	1,362	637	1,373	1,081	1,713	192

Figure 4.5.2 Representative flow cytometric panels showing multiple stained macrophage population stained against M1 (CD86, HLA-DR, CD274, MPO) and M2 (CD206, CD163, IL-10) fluorescent antibodies. *Vertical lines indicate fluorescence thresholds while multiple stained populations found on the right side of the thresholds are indicative of positive staining for the fluorescent labels. Placebo D0 n=9, PCO D0 n=9, Placebo D14 n=9, PCO D14 n=9*

Interestingly, for both CD274 and MPO – both pro-inflammatory indicators – the D0 vs D14 values were inversely correlated in the PCO-treated group only (**Figures 4.5.3 and 4.5.4**). **Figure 4.5.3 and Figure 4.5.4** The PCO group showed a significant Trendline over time that was not evident in the Placebo group.

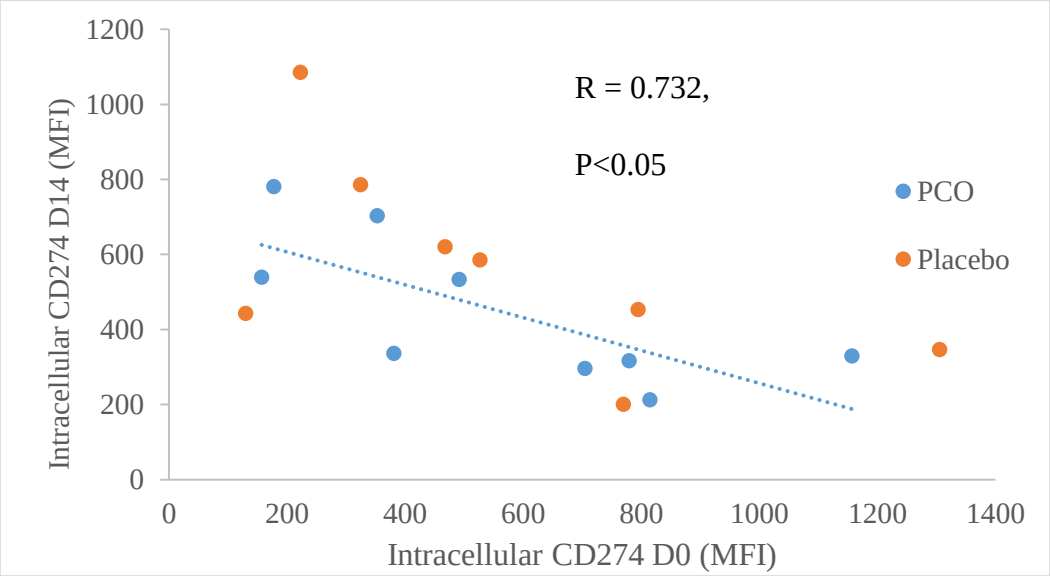


Figure 4.5.3 Relationship between CD274 expression for placebo and PCO treatments with time. *Statistical analysis: Post Hoc Fischer's LSD test (ANOVA) and Pearson P-test. MFI-Median Fluorescent Intensity. Placebo D0 n=9, PCO D0 n=9, Placebo D14 n=9, PCO D14 n=9*

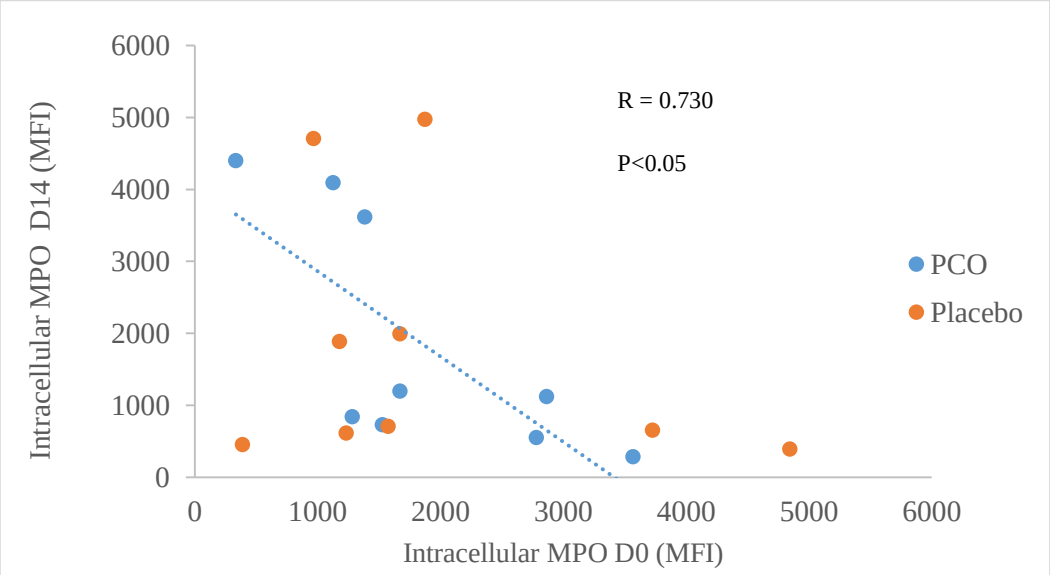


Figure 4.5.4 Relationship between MPO expression for placebo and PCO treatments with time. *MFI-Median Fluorescent Intensity, MPO- Myeloperoxidase. Statistical analysis: Post Hoc Fischer's LSD test (ANOVA) and Pearson P-test. Placebo D0 n=9, PCO D0 n=9, Placebo D14 n=9, PCO D14 n=9*

5. CHAPTER 5: DISCUSSION

Good laboratory and clinical practice guidelines prescribe very specific procedures for the testing of an experimental pharmaceutical drug. After initial proof of concept, potential mechanisms of action of the drug are probed *in vitro,* followed by safety and efficacy studies using suitable animal models. Then, before the drug can be tested in patients with the relevant pathology, the drug is first assessed for effects and/or safety in the absence of disease, in a normally healthy population. The study described in this thesis, present data from such a human trial.

The population investigated in this study – a young, normally healthy population – is not expected to be in a pro-inflammatory state or have high level of cumulative oxidative stress, especially at rest, when blood samples were obtained. Thus, supplementation with an anti-oxidant or anti-inflammatory therapeutic product is unlikely to show major effects in this population. However, despite low statistical power due to large inter- and intra-individual variability in parameters assessed, findings from the study have successfully identified some therapeutic targets that are sufficiently sensitive to provide new information on the mechanisms by which grape-derived polyphenols achieve their previously reported anti-inflammatory effects.

In this chapter, we will first discuss the results obtained from the evaluation of the safety of the grape seed product employed in this study in terms of the risk for bleeding tendencies and general haematology. Next, we will discuss the data pertaining to the potential mechanisms of action of the drug that were elucidated using a novel technique.

5.1 Consumer safety

A direct link between the induction of blood coagulation and the activation of inflammatory processes was reported some years ago (Levi *et al*., 2004). This suggests that these processes are at least to some extent linked, as they both play pivotal roles in the pathogenesis of vascular disease (Levi *et al*., 2004). Thus, it would be difficult to separate these two processes, as many coagulation disorders are associated with inflammatory diseases and vice versa. The two processes are thought to exist in continuum and within three stages; namely,

blood coagulation activation, a decrease in natural anti-coagulant activity and concomitant decrease in fibrinolytic activity. Anti-thrombotic endothelial normal functions are also influenced by inflammation as a loss of homeostasis in the normal coagulation process does not only lead to a stressed thrombotic condition, but also leaves the inflammatory process in a perpetual loop (D'Angelo, 2015).

Also, more recently, anecdotal accounts associating the use of herbal and/or natural supplements with bleeding tendencies have been reported (personal communications between Prof Smith and consumers). To our knowledge, no literature exist on potential effects of PCO on hemostasis. We found only one study investigating product safety. In this study, a clinical trial involving 66 volunteers with moderate or marked breast induration at a mean of 10.8 year post-radiation for early breast cancer, subjects were given 100mg grape seed proanthocyanidin extract (GSPE) three times a day for 6 months. Although no statically significant results were reported in terms of anti-cancer effects, no adverse effects were reported after this relatively prolonged supplementation period (Brooker *et al.*, 2006). More recent work in animals supplemented with PCO for a period of up to two weeks (Myburgh *et al.*, 2012), (Kruger *et al.*, 2014), supports this report of consumer safety, as no adverse effects of PCO supplementation was reported. Although a recent case study involving a 49-year old Caucasian male described the patient presenting with recurrent nausea and vomiting after GSPE consumption, no clinical cause of these symptoms were found after a thorough medical workup. Thus, although these symptoms were reported to subside once supplementation was discontinued (Berry *et al.*, 2016), it cannot be conclusively ascribed to the supplement used. Despite these mostly positive reports, it is probably advisable to err on the side of caution especially in the case of natural products, which often consist of multiple active ingredients. The doses and duration of supplementation are also important factors to consider in this context.

Therefore, in the current study, both the functionality of the intrinsic and extrinsic clotting pathways (see **Figure 6.1**) were assessed after 7 days of PCO or placebo supplementation. . The Prothrombin time (PT) measures the integrity of the extrinsic system (clotting factors X, VII, V, II (Prothrombin) and I (fibrinogen)) as well as considering factors that are common in both systems, while the partial thromboplastin time (PTT) which measures the integrity of the intrinsic system (Factors XII, XI, VIII, and IX), (Hoffbrand *et al.*, 2011). All participants' fibrinogen levels and Prothrombin time were within normal ranges. In addition, an activated partial thromboplastin time (aPTT) test similarly showed no abnormality. Thus, we suggest

that blood coagulation was not adversely affected by the consumption of PCO, at least not to an extent that reached clinical significance within the 7-day period assessed. However, conclusions on long-term effects should not be extrapolated from these data. Given the many positive effects reported for grape-derived products in the literature, long-term safety and drug-interaction studies are warranted.

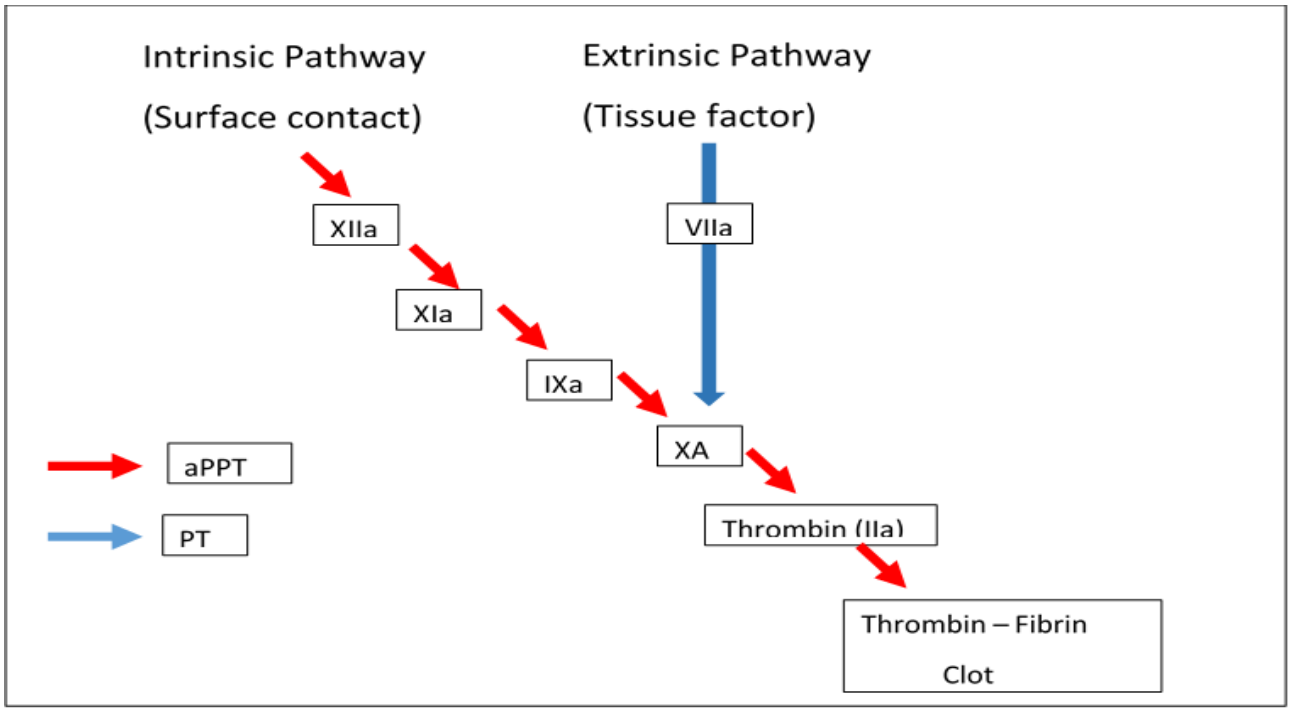


***Figure 6.1**: The Clotting Cascade (Self Illustrated)*

Furthermore, the full blood counts for all individuals (both placebo and PCO groups) remained unchanged and within the normal range. Generally, changes in red blood cell indices – such as microcytosis or an increased red cell distribution width (Allen *et al.*, 2010) – would indicate drug-associated abnormalities in nutrient absorption or even intravascular bleeding (Isik *et al.*, 2012). Also, the latter would often be accompanied by lower platelet counts. No evidence of such adverse effects was reflected in haematology data in the current study. Similarly, the normal and unaffected white blood cell parameters suggests absence of cell proliferation in circulation, which could be indicative of intolerance to or toxicity of the drug/supplement (Guilford *et al.*, 2011). Based on these findings, is we conclude that the PCO supplement employed in this study was safe to consume.

However, at this point, for the sake of being thorough, it is important to note that hemostasis comprises two mechanisms (apart from local vasoconstriction), namely platelet plug

formation and coagulation. Data presented here pertains to coagulation only. In order to assess platelet functionality, a bleeding time test is commonly performed. This entails making a 1mm deep, 10mm long cut into the participant skin, to assess the time taken for a platelet plug to form. Since this test requires some skill and results in permanent scarring on the skin, we decided that the inclusion of this specific test could not be ethically justified.

Returning attention to the links between coagulation and inflammation, it has also been reported that tissue factor plays an important role in inflammation, as the extracellular coagulation signalling pathway is able to trigger an intracellular signalling response. The downstream coagulation factors (VIIa, Xa and thrombin) are pro-inflammatory and are, therefore, capable of causing an inflammatory state by inducing the protease-activated receptors (PAR) found on the surface of cells. PAR activating peptides elicit a broad spectrum of inflammation, which is consistent with the notion of coagulation-dependent inflammation. This activation induces the expression of various inflammatory molecules such as TNF-α, interleukins, and adhesion molecules (ICAM-1 and VCAM-1) (Hoffbrand *et al.*, 2011). The adhesion molecules mediate the adhesion of lymphocytes to the vascular endothelium and may also function in their signalling transduction. When cytokines are activated, ICAM-1 and VCAM-1 expression is upregulated in this pro-inflammatory state and thus lead the risk of atherosclerosis development. However, in the current study, no effect of PCO was evident on either ICAM-1 or VCAM-1 (more on this later), supporting our conclusion on its safety, at least in the context of coagulation.

5.2 Development of novel neutrophil migration model

Normally, *in vitro* neutrophil migration is assessed using a Dunn chamber (Sapey *et al.*, 2011), (Sapey *et al.*, 2014) or microfluidics systems (Irimia *et al.*, 2006), (Irimia, 2010), while videomicroscopy in commonly used for *in vivo* investigations (Germain *et al.*, 2008). However, none of these models allow for immunostaining of cells at the endpoint, as they are all closed systems. Thus, mechanisms cannot be directly linked to functional outcome. Thus, a novel migration assay was developed to overcome this limitation. Eight-well chamber slides were employed, enabling both live cell tracking and post-fixation immunocytochemical staining on the same sample. Neutrophil chemokinesis results reported here, consistently indicate the success of this model and the appropriateness of using fMLP as chemotaxin. To

our knowledge, this technique has not been reported previously. Since PCO had no effect on neutrophil chemokinesis in the healthy population in this study, these results to not inform about the sensitivity of the model in indicating the therapeutic efficacy of anti-inflammatory products. However, the consistent aberration in ROCK-related parameters in the PCO-treated group, suggests that this model may indeed be sufficiently sensitive to elucidate molecular targets of such products. A follow-up study in a population with a more inflammatory phenotype at baseline may provide more information.

Focussing on the actual data generated on potential effects of PCO in this model, as mentioned, no effect on migration was observed for PCO treatment. Although several reports of positive effects of polyphenols and PCO on inflammation exist (Kruger *et al.*, 2012) (Myburgh *et al.*, 2012), there is relative paucity of information on neutrophil migration and chemokinesis after *in vivo* PCO supplementation. In contrast to our current results, an improved neutrophil directional accuracy (total and linear distance) was reported after *in vitro* PCO treatment of cells isolated from an aged population (Petersen *et al.*, 2016). However, these differences in results can be attributed to different effects *in vivo* and *in vitro*. Firstly, neutrophils which are isolated and then treated with PCO are exposed to a more controlled environment (in terms of dose of PCO, time of exposure and potential confounding factors, which allows direct effect of PCO to be seen. Importantly, in an in vitro model, assuming proper laboratory technique and functional equipment, the differences between repeats of any experiment should be fairly small. In contrast, in *in vivo* studies, the net effects of PCO is assessed after the supplement has been metabolised – a process which depends on the inherent metabolic rate of each individual assessed. Furthermore, the neutrophil as the first line of defence is arguably the most excitable immune cell, so that it may be activated for various reasons unrelated to the study intervention. Thus, inter- and intra-individual variations may mask effects, adding to the complexity of interpreting data from a model such as the one employed here. This variability was indeed noted in both the PCO group and placebo group, as well as for both groups over time. In order to better understand the variation, a reliability value calculation was performed on both total and linear distance, both between individuals and for the same individuals over time. Briefly, reliability was calculated as follows:

The inter-individual repeatability value was 0.61 and 0.43 for total and linear distance respectively, and the intra-individual repeatability value 0.39 and 0.57. This implies that

inter-person variation contributed to 61% and 43% of the variability in current data. Statistical power could therefore be improved by addition of more subjects. A subsequent power analysis indicated that more than 80 subjects would be required to provide sufficient statistical power – a number that is not feasible, given the cost of analyses. Furthermore, given the fact that day-to-day variation is shown to contribute 39% and 57% of variability for total and linear distance respectively, even data from a bigger group is likely to be complex to interpret in a longitudinal study design. Rather, we recommend that in vivo studies should either be of cross-sectional design – i.e. focus on acute effects on potential molecular targets – or that a very controlled clinical trial should be conducted, during which not only food intake, but also exposure to potential antigens, can be limited. Alternatively, a population with a poorer inflammatory profile may be more ideal to illustrate anti-inflammatory effects. Although no significant effect was seen in terms of neutrophil movement parameters, given this variability, it does not exclude molecular effect. This is discussed in the next sections.

5.3 Potential Molecular Mechanisms affected

Plasma levels of soluble adhesion molecules is the more commonly used protocol for assessing cellular adhesion dynamics in the recent literature. An obvious limitation of this approach is that the source of adhesion molecules in plasma cannot be deduced from this, since the shedding of expression markers can be attributed to various cell types. Very little literature is available on neutrophil expression of adhesion molecules. One study investigating the ability of IL-4 to induce adherence in neutrophils included assessment of cellular expression of VCAM-1 and ICAM-1 in neutrophils after the stimulation. They found IL-4 had no effect on neutrophil adherence and that only VCAM-1 expression was induced but not at such a rate that it affected the expression of other adhesion molecules (Schleimer *et al.*, 1992) .Furthermore, we could find no information in literature on the cellular expression of adhesion molecules, such as CD66b, ICAM-1 or VCAM-1, in neutrophils under unstimulated conditions (i.e. basal expression). Hence, we assessed the expression of these surface and cytoplasmic adhesion molecules in neutrophils, to determine the cell-specific effects of PCO supplementation in healthy individuals. Results obtained from the present study indicated that PCO has no effect on intracellular expression of CD66b, ICAM-1 or VCAM-1. Although one would expect an anti-inflammatory product to exhibit significant modulatory effects on adhesion, our result is actually supported by the data reported by

Schleimer et al. (1992), since they also found no change after treatment with IL-4, which is also a known anti-inflammatory modality (Boyle *et al.*, 1999). In contrast, an *in vitro* study on the effects of red grape polyphenols on some of these markers (Noratto *et al.*, 2011), reported decreased ICAM-1 protein and gene expression. However, this effect of the polyphenols were only evident after stimulation of the (HUVEC) cells to lipopolysaccharide (LPS), which is a known up-regulator of these adhesion molecules (Thornhill *et al.*, 1990), (Briscoe *et al.*, 1992), (Huang *et al.*, 1996). Together, these results suggest that assessment of anti-inflammatory effects under basal conditions is not reflected by cellular adhesion protein expression.

However, criticism against the use of soluble adhesion molecule levels have also been published. For example, although upregulated soluble adhesion molecule levels had been reported in extreme inflammatory models, such as Gave opthlamopathy and thyroid disease (Heufelder *et al.*, 1993), as well as in rheumatoid arthritis (Cush *et al.*, 1993), soluble adhesion molecule shifts were not evident at the point of relapse in Wegener's granulomatosis – a somewhat milder inflammatory condition (Stegeman *et al.*, 1994). Thus, we propose that the best model to use would be an *in vivo* model of supplementation, with effects of supplementation assessed in a cell-specific manner rather than assessment of soluble adhesion molecules. However, it would be important to conduct these assessments *after* an *in vitro* application of an appropriate pro-inflammatory stimulus such as LPS, in cases where only mild or low grade inflammation exist. A more comprehensive study, assessing both soluble and neutrophil-associated adhesion molecule levels, both under basal and stimulated conditions, could shed more light on this issue.

Nevertheless, the data generated in this study may provide a basis for the establishment of a reference range for CD66B, ICAM-1 and VCAM-1 expression for this technique used in normal, healthy neutrophil populations, as assessed by flow cytometry. Also, it provides evidence of large variations not just between participants, but also intra-individual, day-to-day variations at basal level under unstimulated conditions, which may explain why the positive effects of PCO reported from more controlled study designs and models, have not been duplicated in the current study.

5.4 Neutrophil polarization

As previously explained, neutrophil polarization is a process that causes neutrophils to undergo a mechanical shape change (elongation) which demonstrate a clear leading and tail end of the cell (Shi *et al.*, 2009). To understand the possible mechanisms involved within the leading and trailing edge, the expressions of PI3K and ROCK in neutrophils were investigated.

Similar to the results for adhesion molecule expression, neutrophil PI3K expression was highly variable between neutrophils in any single sample, between individuals, and also over time – these variations were also independent of stimulation. For example, in the placebo group, median fluorescence intensity after fMLP stimulation was 0.89 on day 0, but almost double on day 14, at 1.61. While the reason for this similar trend in all groups are not clear, it is of interest that in the PCO group, this increase over time seemed to be attenuated, although not reaching statistical significance. Thus, PCO could potentially act as a modulator for PI3K, thereby preventing over-activation of inflammation. PI3K is known to be found in the leading edge and also responsible for the synthesis of PIP3 (Billadeau, 2008), (Heit *et al.*, 2008). PIP3 accumulation in the leading edge is seen as an early and noticeable occurrence once cell polarization is initiated as a result of chemoattractant stimulation (Servant *et al.*, 2000) (Hawkins *et al.*, 2010). However, the over production of PIP3 in the rear has been linked to a disruption of the PIP3-polarity gradient (Mondal *et al.*, 2012) and thus affecting neutrophil directionality. Our data may thus suggest that in this context, PCO – by preventing over-activation of PI3K – could stabilise the expression of PIP3 in the rear of the cell, thereby improving directional accuracy. However, given the large variability of this parameter in an *in vivo* model, this potential mechanism should be investigated in more detail in a more controlled *in vitro* experiment, before firm conclusions may be made.

Our interpretation above, which suggests that PCO may have its effect by separating localisation sites of PI3K (to the front) and ROCK (to the rear) in a migrating cell, is supported by ROCK expression data. As for PI3K, ROCK expression was once again variable, but also showed similar increases over time, again under both stimulated and unstimulated conditions. Again, as reported for PI3K, the only disturbance in this trend was seen in the PCO stimulated group, which showed a ≈25% decrease in median fluorescent intensity from 0.44 MFI on day 0 to 0.33 on day 14 – unlike the PI3K result, which lacked

statistical significance, ROCK expression showed a more significant tendency for treatment-time-stimulation interaction.

In terms of the literature on changes in ROCK expression, a decrease has been linked to increased recruitment and migration of neutrophils (Vemula *et al.*, 2010). In contrast, the loss of ROCK has been associated with neutrophil tail retraction defects (Pestonjamasp *et al.*, 2006), (Worthylake *et al.*, 2001). This suggests that an optimal balance in ROCK expression is required for optimal migration. We suggest that PCO may act as such an "optimising modulator" of ROCK. This is vital to the entire neutrophil migratory mechanism, as ROCK is known to regulate PTEN whose role is to dephosphorylate PIP3 to PIP2 and maintain the anterior-posterior PIP3 gradient (Mondal *et al.*, 2012), which is vital for maintaining directional accuracy (Billadeau, 2008), (Heit *et al.*, 2008). The amount of PIP3 as well as the localization within the cell is positively associated with neutrophil migration and the recruitment of neutrophils (Vanhaesebroeck *et al.*, 2001), (Hawkins *et al.*, 2006). Our data, together with the known anti-inflammatory effect of PCO from previous studies, suggests that PTEN could also be modulated and could be implicated of as potential mechanism of action for PCO. An assessment of all these parameters simultaneously will further elucidate PCO effects on this particular mechanism. However, it is clear from the literature that this process is highly complex and extremely sensitive to maintain.

More interesting facts in support of our interpretation of ROCK and PI3K expression modulation by PCO, came to light when, in addition to expression levels, co-localisation of PI3K and ROCK was assessed.

Firstly, current results suggest that a greater extent of co-localization of PI3K and ROCK may occur under stimulated conditions than in unstimulated conditions, with relative ROCK co-localisation to PI3K more significantly increased (≈25%, $P<0.05$) than relative PI3K co-localisation with ROCK. As no literature is available on PI3K and ROCK expression on actively migrating neutrophils, placing our results (obtained using our novel approach), into context proved challenging. Nevertheless, this result in in line with our interpretation of the data on adhesion molecules – i.e. that the effects of PCO should be studied only after stimulation.

Secondly, PCO treatment seemed to limit the increase in ROCK-PI3K co-localisation after stimulation, with ROCK seemingly the more sensitive target, as no change in total expression and/or co-localization of PI3K with ROCK was seen. This result importantly supports our

interpretation that PCO may not necessarily affect total ROCK or PI3K expression levels, but may rather optimise the sites in the cell where these are localised. The decrease in ROCK localisation with PI3K may indeed point towards a PCO-associated polarisation of the cell to have ROCK focused in the rear and PI3K focused in the leading edge of the cell.

Another consideration in data interpretation – especially given the illustrated importance of stimulation – is the timing of assessments after fMLP stimulation. In the current study, assessments were made at a time point 40 minutes after stimulation. In the literature, PTEN – which functions in the rear of the cell in association with ROCK – was demonstrated to be focused in the rear of murine neutrophils in less than 3 minutes after stimulation, with the effect of stimulation subsiding somewhat by 5 minutes, when multiple pseudopod formation was evident (Subramanian *et al.*, 2007). Thus, our results on co-localisation suggests firstly that although early timepoints illustrates the initiation of migration, the role players in cell polarisation can still be effectively investigated at a much later time point. This fact enables the design of studies assessing both chemokinesis and mechanisms for chemokinesis in the same cells – validating our approach. Secondly, current data indicates that the effect of PCO was maintained at least for 40 minutes after stimulation. A follow-up study using our novel model, but incorporating more frequent time points, may be useful to fully elucidate not only effects of PCO, but also the time courses involved in migratory mechanisms, especially under conditions of sustained stimulation.

On a more clinical note, our results also support previous work by our group in the context of muscle injury. ROCK is associated with limiting excessive neutrophil recruitment (Vemula *et al.*, 2010) and we have previously shown PCO supplementation to decrease neutrophil recruitment towards site of injury after experimental contusion damage to skeletal muscle, which resulted in less secondary damage (Smith *et al.*, 2008) (Myburgh *et al.*, 2012) (Kruger *et al.*, 2014). Our data thus suggests that PCO-associated optimisation of ROCK localisation relative to PI3K, may be the mechanism through which this *in vivo* result was achieved.

5.5 Macrophage phenotype

Apart from neutrophils, macrophages also have an important role to play in the inflammatory process. They play a direct role as phagocytes, and more relevant to this thesis, macrophages are known to exist within two phenotypes (M1 and M2) and secrete a variety of chemokines,

each befitting their own observable characteristics (Mia *et al.*, 2014) – these factors may affect neutrophil migration and differ between macrophage phenotypes (Mia *et al.*, 2014). As mentioned in the literature review, phenotype shifts after an intervention is most often determined in response to a stimulus (Tan *et al.*, 2016). However, apart from the fact that such an experimental approach would be financially unfeasible, the aim in the current study was not to determine whether immune response is altered in reaction to a challenge; rather, the aim was to determine whether basal phenotype profiles would be adapted in response to PCO. This is due to previous studies performed by our group (rat models) that demonstrated a basal phenotypic shift after PCO consumption with the M2 phenotype predominating (Myburgh *et al.*, 2012). An earlier phenotypic switch from M1 macrophages to M2 macrophages is a desired effect, not only because M2 macrophages are known to possess anti-inflammatory properties, but also because they cannot cross the endothelial membranes (Arnold *et al.*, 2007). This in turn assists in faster clearance of inflammation (Mia *et al.*, 2014).

Current data indicates large variations between individuals and intra-individual day-to-day variations with no apparent effect of PCO. There are a few explanations for this: Firstly, PCO could have no effect on target macrophages, although this is unlikely, given the reports of a phenotype change after supplementation in rats. Secondly, the population used could not be suitable or sensitive enough to see a desired effect. This is likely, since a healthy, young population are at relatively low risk for low grade inflammation in the absence of a stimulus. Thirdly, no stimulus was used to induce a phenotype switch as discussed previously. Again, perhaps a more severe model, e.g. arthritis or advanced age, would enable interpretation on a potential PCO-induced phenotype switch towards a relatively more anti-inflammatory profile. In support of this, during disease progression of atherosclerosis, the levels and intensities of various signals in the microenvironment have been reported to cause macrophage phenotypic marker expressions to vary (Leitinger *et al.*, 2013), (Geissmann *et al.*, 2010) This offers a more plausible explanation for the variance seen within this data set, especially for the basal levels captured.

Interestingly, a significant inverse correlation existed between D0 and D14 macrophage expression levels of the M1 markers CD274 and MPO within the PCO group only. This suggests that PCO could possess the potential of being able to modulate M1 macrophage phenotype; thus, being effectively able to decrease relatively higher expression levels while increasing relatively lower levels. This was seen at basal levels and barring stimulus, which is

the first proof of a direct effect of PCO on human macrophages. Although literature has shown that mild intervention of antioxidant treatment can induce phenotypic shift of macrophages under pro-inflammatory conditions (Myburgh *et al.*, 2012), this study offers a novel approach by including basal level expression of macrophage phenotypes under unstimulated conditions. Given the CD274 and MPO data, it illustrates that these markers may be deemed sensitive enough to provide proof of a therapeutic anti-inflammatory effect.

Both CD274 and MPO are known to be M1 markers and are able to secrete pro-inflammatory cytokines as well as ROS. In excess, ROS can be extremely detrimental to the immune cell itself (Tan *et al.*, 2016). Hence this finding being of importance, as it assist in the modulation of oxidative stress. In support of MPO being modulated in rats, manipulation of this phenotype shift has been proposed as therapeutic avenue and has been investigated to some extent (Kruger *et al.*, 2012). Here we demonstrate that PCO can modulate MPO at basal levels in humans and that a direct effect is seen. The fact that this inverse correlation was not present in the placebo group, excludes the possibility that these correlations were as result of normal daily variations in immune activation.

5.6 Conclusions and recommendations

In conclusion, we have developed a protocol for simultaneous assessment of neutrophil chemokinetic movement toward a chemotaxin and the cellular role players orchestrating this highly complex, synchronised event. This novel model was validated in terms of sensitivity and choice of chemotaxin, by the significant increase in the co-localization of ROCK with PI3K after fMLP stimulation.

In terms of the supplement investigated, data indicates that in the absence of extreme pro-inflammatory conditions *in vivo*, experimental stimulation of isolated neutrophils is required to accurately reflect effects of supplements or anti-inflammatory modalities. Secondly, we have identified molecular targets of PCO, namely the PI3K-PTEN-ROCK system. Furthermore, result indicate that rather than hugely affecting actual expression of these proteins involved in cell polarisation, PCO may optimise the location of these molecules within the activated cell, to prevent opposing functions of different role players from compromising the front-rear synchronisation required for optimal cell movement.

Our results are thus in line with our hypothesis. Given the requirement for stimulation and the highly variable nature of the parameters in an in vivo context, we propose that mechanistic studies be limited to in vitro models. Alternatively, more extreme models of inflammation – such as aged populations or those suffering from inflammatory disease – may be more suitable to illustrate PCO-induced anti-inflammatory effects over and above the normal variation.

6. REFERENCES

Ahmad, N., Gupta, S. and Mukhtar, H. (2000) 'Green tea polyphenol epigallocatechin-3-gallate differentially modulates nuclear factor κB in cancer cells versus normal cells', *Archives of biochemistry and biophysics*. Elsevier, 376(2), pp. 338–346.

Allen, L. A., Felker, G. M., Mehra, M. R., Chiong, J. R., Dunlap, S. H., Ghali, J. K., Lenihan, D. J., Oren, R. M., Wagoner, L. E. and Schwartz, T. A. (2010) 'Validation and potential mechanisms of red cell distribution width as a prognostic marker in heart failure', *Journal of cardiac failure*. Elsevier, 16(3), pp. 230–238.

Andrade, J. M. M., Aboy, A. L., Apel, M. A., Raseira, M. C. B., Pereira, J. F. M. and Henriques, A. T. (2011) 'Phenolic composition in different genotypes of Guabiju fruits (Myrcianthes pungens) and their potential as antioxidant and antichemotactic agents', *Journal of food science*. Wiley Online Library, 76(8), pp. C1181–C1187.

Arnold, L., Henry, A., Poron, F., Baba-Amer, Y., Van Rooijen, N., Plonquet, A., Gherardi, R. K. and Chazaud, B. (2007) 'Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis', *The Journal of experimental medicine*. Rockefeller Univ Press, 204(5), pp. 1057–1069.

Bagchi, D., Bagchi, M., Stohs, S. J., Das, D. K., Ray, S. D., Kuszynski, C. A., Joshi, S. S. and Pruess, H. G. (2000) 'Free radicals and grape seed proanthocyanidin extract: importance in human health and disease prevention', *Toxicology*. Elsevier, 148(2), pp. 187–197.

Balasubramanian, S., Efimova, T. and Eckert, R. L. (2002) 'Green tea polyphenol stimulates a Ras, MEKK1, MEK3, and p38 cascade to increase activator protein 1 factor-dependent involucrin gene expression in normal human keratinocytes', *Journal of Biological Chemistry*. ASBMB, 277(3), pp. 1828–1836.

Berry, A. C., Nakshabendi, R., Abidali, H., Atchaneeyasakul, K., Dholaria, K., Johnson, C., Kishore, V. A. and Baltz, A. C. (2016) 'Adverse Effects of Grape Seed Extract Supplement: A Clinical Case and Long-Term Follow-Up', *Journal of dietary supplements*. Taylor & Francis, 13(2), pp. 232–235.

Berzat, A. and Hall, A. (2010) 'Cellular responses to extracellular guidance cues', *The EMBO Journal*. EMBO Press, 29(16), pp. 2734–2745.

Billadeau, D. D. (2008) 'PTEN gives neutrophils direction', *Nature immunology*. Nature Publishing Group, 9(7), pp. 716–718.

Boyle, D. L., Nguyen, K. H., Zhuang, S., Shi, Y., McCormack, J. E., Chada, S. and Firestein, G. S. (1999) 'Intra-articular IL-4 gene therapy in arthritis: anti-inflammatory effect and enhanced Th2 activity', *Gene therapy*, 6(12), pp. 1911–1918.

Briscoe, D. M., Cotran, R. S. and Pober, J. S. (1992) 'Effects of tumor necrosis factor, lipopolysaccharide, and IL-4 on the expression of vascular cell adhesion molecule-1 in vivo. Correlation with CD3+ T cell infiltration.', *The Journal of Immunology*. Am Assoc Immnol, 149(9), pp. 2954–2960.

Brooker, S., Martin, S., Pearson, A., Bagchi, D., Earl, J., Gothard, L., Hall, E., Porter, L. and Yarnold, J. (2006) 'Double-blind, placebo-controlled, randomised phase II trial of IH636 grape seed proanthocyanidin extract (GSPE) in patients with radiation-induced breast induration', *Radiotherapy and Oncology*. Elsevier, 79(1), pp. 45–51.

Cartea, M. E., Francisco, M., Soengas, P. and Velasco, P. (2010) 'Phenolic compounds in Brassica vegetables', *Molecules*. Molecular Diversity Preservation International, 16(1), pp. 251–280.

Chao, P.-Y., Huang, Y.-P. and Hsieh, W.-B. (2013) 'Inhibitive effect of purple sweet potato leaf extract and its components on cell adhesion and inflammatory response in human aortic endothelial cells', *Cell adhesion & migration*. Taylor & Francis, 7(2), pp. 237–245.

Chazaud, B., Sonnet, C., Lafuste, P., Bassez, G., Rimaniol, A.-C., Poron, F., Authier, F.-J., Dreyfus, P. A. and Gherardi, R. K. (2003) 'Satellite cells attract monocytes and use macrophages as a support to escape apoptosis and enhance muscle growth', *The Journal of cell biology*. Rockefeller Univ Press, 163(5), pp. 1133–1143.

Chen, C.-C., Chow, M.-P., Huang, W.-C., Lin, Y.-C. and Chang, Y.-J. (2004) 'Flavonoids inhibit tumor necrosis factor-α-induced up-regulation of intercellular adhesion molecule-1 (ICAM-1) in respiratory epithelial cells through activator protein-1 and nuclear factor-κB: structure-activity relationships', *Molecular Pharmacology*. ASPET, 66(3), pp. 683–693.

Chen, X., Hasuma, T., Yano, Y., Yoshimata, T., Morishima, Y., Wang, Y. and Otani, S. (1996) 'Inhibition of farnesyl protein transferase by monoterpene, curcumin derivatives and gallotannin.', *Anticancer research*, 17(4A), pp. 2555–2564.

Ciccia, F., Alessandro, R., Rodolico, V., Guggino, G., Raimondo, S., Guarnotta, C., Giardina,

A., Sireci, G., Campisi, G. and De Leo, G. (2013) 'IL-34 is overexpressed in the inflamed salivary glands of patients with Sjögren's syndrome and is associated with the local expansion of pro-inflammatory CD14brightCD16+ monocytes', *Rheumatology*. Br Soc Rheumatology, 52(6), pp. 1009–1017.

Cush, J. J., Rothlein, R., Lindsley, H. B., Mainolfi, E. A. and Lipsky, P. E. (1993) 'Increased levels of circulating intercellular adhesion molecule 1 in the sera of patients with rheumatoid arthritis', *Arthritis & Rheumatism*. Wiley Online Library, 36(8), pp. 1098–1102.

D'Angelo, G. (2015) 'Inflammation and coagulation: a "continuum" between coagulation activation and prothrombotic state', *J Blood Disord*, 2(1), p. 1023.

Daniel, J. L., Dangelmaier, C., Jin, J., Kim, Y. B. and Kunapuli, S. P. (1999) 'Role of intracellular signaling events in ADP-induced platelet aggregation', *Thrombosis and haemostasis*. Schattauer Publishers, 82(4), pp. 1322–1326.

DeMarco, R. A., Fink, M. P. and Lotze, M. T. (2005) 'Monocytes promote natural killer cell interferon gamma production in response to the endogenous danger signal HMGB1', *Molecular immunology*. Elsevier, 42(4), pp. 433–444.

Derlindati, E., Dall'Asta, M., Ardigò, D., Brighenti, F., Zavaroni, I., Crozier, A. and Del Rio, D. (2012) 'Quercetin-3-O-glucuronide affects the gene expression profile of M1 and M2a human macrophages exhibiting anti-inflammatory effects', *Food & function*. Royal Society of Chemistry, 3(11), pp. 1144–1152.

Dinkova-Kostova, A. T., Liby, K. T., Stephenson, K. K., Holtzclaw, W. D., Gao, X., Suh, N., Williams, C., Risingsong, R., Honda, T. and Gribble, G. W. (2005) 'Extremely potent triterpenoid inducers of the phase 2 response: correlations of protection against oxidant and inflammatory stress', *Proceedings of the National Academy of Sciences of the United States of America*. National Acad Sciences, 102(12), pp. 4584–4589.

Donnelly, L. E., Newton, R., Kennedy, G. E., Fenwick, P. S., Leung, R. H. F., Ito, K., Russell, R. E. K. and Barnes, P. J. (2004) 'Anti-inflammatory effects of resveratrol in lung epithelial cells: molecular mechanisms', *American Journal of Physiology-Lung Cellular and Molecular Physiology*. Am Physiological Soc, 287(4), pp. L774–L783.

Engelbrecht, A.-M., Mattheyse, M., Ellis, B., Loos, B., Thomas, M., Smith, R., Peters, S., Smith, C. and Myburgh, K. (2007) 'Proanthocyanidin from grape seeds inactivates the PI3-kinase/PKB pathway and induces apoptosis in a colon cancer cell line', *Cancer letters*.

Elsevier, 258(1), pp. 144–153.

Erlandsson-Harris, H. and Raucci, A. (2006) 'Alarmin (g) news about danger', in *Workshop on Innate Danger Signals and HMGB1. EMBO Rep*, pp. 774–778.

Farges, M.-C., Balcerzak, D., Fisher, B. D., Attaix, D., Béchet, D., Ferrara, M. and Baracos, V. E. (2002) 'Increased muscle proteolysis after local trauma mainly reflects macrophage-associated lysosomal proteolysis', *American Journal of Physiology-Endocrinology And Metabolism*. Am Physiological Soc, 282(2), pp. E326–E335.

Ferguson, G. J., Milne, L., Kulkarni, S., Sasaki, T., Walker, S., Andrews, S., Crabbe, T., Finan, P., Jones, G. and Jackson, S. (2007) 'PI (3) Kγ has an important context-dependent role in neutrophil chemokinesis', *Nature cell biology*. Nature Publishing Group, 9(1), pp. 86–91.

Foxman, E. F., Campbell, J. J. and Butcher, E. C. (1997) 'Multistep navigation and the combinatorial control of leukocyte chemotaxis', *The Journal of cell biology*. Rockefeller Univ Press, 139(5), pp. 1349–1360.

Gambardella, L. and Vermeren, S. (2013) 'Molecular players in neutrophil chemotaxis--focus on PI3K and small GTPases.', *Journal of leukocyte biology*, 94(4), pp. 603–12. doi: 10.1189/jlb.1112564.

Garbacki, N., Tits, M., Angenot, L. and Damas, J. (2004) 'Inhibitory effects of proanthocyanidins from Ribes nigrum leaves on carrageenin acute inflammatory reactions induced in rats', *BMC pharmacology*. BioMed Central Ltd, 4(1), p. 25.

Gaziano, J. M., Manson, J. E., Branch, L. G., Colditz, G. A., Willett, W. C. and Buring, J. E. (1995) 'A prospective study of consumption of carotenoids in fruits and vegetables and decreased cardiovascular mortality in the elderly', *Annals of epidemiology*. Elsevier, 5(4), pp. 255–260.

Geissmann, F., Gordon, S., Hume, D. A., Mowat, A. M. and Randolph, G. J. (2010) 'Unravelling mononuclear phagocyte heterogeneity', *Nature Reviews Immunology*. Nature Publishing Group, 10(6), pp. 453–460.

Germain, R. N., Bajénoff, M., Castellino, F., Chieppa, M., Egen, J. G., Huang, A. Y. C., Ishii, M., Koo, L. Y. and Qi, H. (2008) 'Making friends in out-of-the-way places: how cells of the immune system get together and how they conduct their business as revealed by intravital imaging', *Immunological reviews*. Wiley Online Library, 221(1), pp. 163–181.

Gordon, S. (2003) 'Alternative activation of macrophages', *Nature reviews immunology*. Nature Publishing Group, 3(1), pp. 23–35.

Gordon, S. (2012) 'Innate immune functions of macrophages in different tissue environments', *Journal of innate immunity*. Karger Publishers, 4(5–6), pp. 409–410.

Gorina, R., Lyck, R., Vestweber, D. and Engelhardt, B. (2014) 'β2 integrin-mediated crawling on endothelial ICAM-1 and ICAM-2 is a prerequisite for transcellular neutrophil diapedesis across the inflamed blood-brain barrier.', *Journal of immunology (Baltimore, Md.: 1950)*, 192(1), pp. 324–337.

Guilford, J. M. and Pezzuto, J. M. (2011) 'Wine and health: a review', *American Journal of Enology and Viticulture*. Am Soc Enol Viticulture, p. ajev-2011.

Hawkins, P. T., Anderson, K. E., Davidson, K. and Stephens, L. R. (2006) 'Signalling through Class I PI3Ks in mammalian cells', *Biochemical Society Transactions*. Portland Press Limited, 34(5), pp. 647–662.

Hawkins, P. T., Stephens, L. R., Suire, S. and Wilson, M. (2010) 'PI3K signaling in neutrophils', in *Phosphoinositide 3-kinase in Health and Disease*. Springer, pp. 183–202.

He, F. J., Nowson, C. A. and MacGregor, G. A. (2006) 'Fruit and vegetable consumption and stroke: meta-analysis of cohort studies', *The Lancet*. Elsevier, 367(9507), pp. 320–326.

Heasman, S. J. and Ridley, A. J. (2011) 'Multiple roles for RhoA during T cell transendothelial migration', *Small GTPases*. Taylor & Francis.

Heit, B., Robbins, S. M., Downey, C. M., Guan, Z., Colarusso, P., Miller, B. J., Jirik, F. R. and Kubes, P. (2008) 'PTEN functions to'prioritize'chemotactic cues and prevent'distraction'in migrating neutrophils', *Nature immunology*. Nature Publishing Group, 9(7), pp. 743–752.

Heit, B., Tavener, S., Raharjo, E. and Kubes, P. (2002) 'An intracellular signaling hierarchy determines direction of migration in opposing chemotactic gradients', *The Journal of cell biology*. Rockefeller Univ Press, 159(1), pp. 91–102.

Heufelder, A. E. and Bahn, R. S. (1993) 'Soluble intercellular adhesion molecule-1 (sICAM-1) in sera of patients with Graves' ophthalmopathy and thyroid diseases', *Clinical & Experimental Immunology*. Wiley Online Library, 92(2), pp. 296–302.

Hillgruber, C., Pöppelmann, B., Weishaupt, C., Steingräber, A. K., Wessel, F., Berdel, W. E.,

Gessner, J. E., Ho-Tin-Noé, B., Vestweber, D. and Goerge, T. (2015) 'Blocking neutrophil diapedesis prevents hemorrhage during thrombocytopenia', *The Journal of experimental medicine*. Rockefeller Univ Press, 212(8), pp. 1255–1266.

Hodgetts, S., Radley, H., Davies, M. and Grounds, M. D. (2006) 'Reduced necrosis of dystrophic muscle by depletion of host neutrophils, or blocking TNFα function with Etanercept in mdx mice', *Neuromuscular Disorders*. Elsevier, 16(9), pp. 591–602.

Hoeller, O. and Kay, R. R. (2007) 'Chemotaxis in the absence of PIP3 gradients', *Current Biology*. Elsevier, 17(9), pp. 813–817.

Hoffbrand, V. and Moss, P. A. H. (2011) *Essential haematology*. John Wiley & Sons.

Huang, G. T., Eckmann, L., Savidge, T. C. and Kagnoff, M. F. (1996) 'Infection of human intestinal epithelial cells with invasive bacteria upregulates apical intercellular adhesion molecule-1 (ICAM)-1) expression and neutrophil adhesion.', *Journal of Clinical Investigation*. American Society for Clinical Investigation, 98(2), p. 572.

Irimia, D. (2010) 'Microfluidic technologies for temporal perturbations of chemotaxis', *Annual review of biomedical engineering*. NIH Public Access, 12, p. 259.

Irimia, D., Liu, S.-Y., Tharp, W. G., Samadani, A., Toner, M. and Poznansky, M. C. (2006) 'Microfluidic system for measuring neutrophil migratory responses to fast switches of chemical gradients', *Lab on a Chip*. Royal Society of Chemistry, 6(2), pp. 191–198.

Isik, T., Uyarel, H., Tanboga, I. H., Kurt, M., Ekinci, M., Kaya, A., Ayhan, E., Ergelen, M., Bayram, E. and Gibson, C. M. (2012) 'Relation of red cell distribution width with the presence, severity, and complexity of coronary artery disease', *Coronary artery disease*. LWW, 23(1), pp. 51–56.

Jang, J. H., Yang, E. S., Min, K.-J. and Kwon, T. K. (2012) 'Inhibitory effect of butein on tumor necrosis factor-α-induced expression of cell adhesion molecules in human lung epithelial cells via inhibition of reactive oxygen species generation, NF-κB activation and Akt phosphorylation', *International journal of molecular medicine*. Spandidos Publications, 30(6), pp. 1357–1364.

Järvinen, T. A. H., Järvinen, T. L. N., Kääriäinen, M., Kalimo, H. and Järvinen, M. (2005) 'Muscle injuries biology and treatment', *The American journal of sports medicine*. American Orthopaedic Society for Sports Medicine, 33(5), pp. 745–764.

Jensen, T. O., Schmidt, H., Møller, H. J., Donskov, F., Høyer, M., Sjoegren, P., Christensen, I. J. and Steiniche, T. (2012) 'Intratumoral neutrophils and plasmacytoid dendritic cells indicate poor prognosis and are associated with pSTAT3 expression in AJCC stage I/II melanoma', *Cancer*. Wiley Online Library, 118(9), pp. 2476–2485.

Jung, J., Ko, S. H., Yoo, D. Y., Lee, J. Y., Kim, Y., Choi, S. M., Kang, K. K., Yoon, H. J., Kim, H. and Youn, J. (2012) '5, 7-Dihydroxy-3, 4, 6-trimethoxyflavone inhibits intercellular adhesion molecule 1 and vascular cell adhesion molecule 1 via the Akt and nuclear factor-κB-dependent pathway, leading to suppression of adhesion of monocytes and eosinophils to bronchial epi', *Immunology*. Wiley Online Library, 137(1), pp. 98–113.

Kalfin, R., Righi, A., Del Rosso, A., Bagchi, D., Generini, S., Guiducci, S., Matucci Cerinic, M. and Das, D. K. (2002) 'Activin, a grape seed-derived proanthocyanidin extract, reduces plasma levels of oxidative stress and adhesion molecules (ICAM-1, VCAM-1 and E-selectin) in systemic sclerosis', *Free radical research*. Taylor & Francis, 36(8), pp. 819–825.

Kalkman, H. O. and Feuerbach, D. (2016) 'Antidepressant therapies inhibit inflammation and microglial M1-polarization', *Pharmacology & therapeutics*. Elsevier, 163, pp. 82–93.

Kamakura, S., Nomura, M., Hayase, J., Iwakiri, Y., Nishikimi, A., Takayanagi, R., Fukui, Y. and Sumimoto, H. (2013) 'The cell polarity protein mInsc regulates neutrophil chemotaxis via a noncanonical G protein signaling pathway', *Developmental cell*. Elsevier, 26(3), pp. 292–302.

Kaneider, N. C., Mosheimer, B., Reinisch, N., Patsch, J. R. and Wiedermann, C. J. (2004) 'Inhibition of thrombin-induced signaling by resveratrol and quercetin: effects on adenosine nucleotide metabolism in endothelial cells and platelet–neutrophil interactions', *Thrombosis research*. Elsevier, 114(3), pp. 185–194.

Kim, D.-C., Lee, W. and Bae, J.-S. (2011) 'Vascular anti-inflammatory effects of curcumin on HMGB1-mediated responses in vitro', *Inflammation Research*. Springer, 60(12), pp. 1161–1168.

Kouwenhoven, M., Teleshova, N., Özenci, V., Press, R. and Link, H. (2001) 'Monocytes in multiple sclerosis: phenotype and cytokine profile', *Journal of neuroimmunology*. Elsevier, 112(1), pp. 197–205.

Kruger, M. J., Myburgh, K. H. and Smith, C. (2014) 'Contusion injury with chronic in vivo polyphenol supplementation: Leukocyte responses', *Medicine and Science in Sports and*

Exercise, 46(2), pp. 225–231. doi: 10.1249/MSS.0b013e3182a4e754.

Kruger, M. J. and Smith, C. (2012) 'Postcontusion polyphenol treatment alters inflammation and muscle regeneration', *Medicine and Science in Sports and Exercise*, 44(5), pp. 872–880. doi: 10.1249/MSS.0b013e31823dbff3.

Kümper, S., Mardakheh, F. K., McCarthy, A., Yeo, M., Stamp, G. W., Paul, A., Worboys, J., Sadok, A., Jørgensen, C. and Guichard, S. (2016) 'Rho-associated kinase (ROCK) function is essential for cell cycle progression, senescence and tumorigenesis', *Elife*. eLife Sciences Publications Limited, 5, p. e12203.

Kuntz, S. and Kunz, C. (2014) 'Extracts from Brassica oleracea L. convar. acephala var. sabellica inhibit TNF-α stimulated neutrophil adhesion in vitro under flow conditions', *Food & function*. Royal Society of Chemistry, 5(6), pp. 1082–1090.

Kusters, D. H. M., Tegtmeier, J., Schurgers, L. J. and Reutelingsperger, C. P. M. (2012) 'Molecular imaging to identify the vulnerable plaque—from basic research to clinical practice', *Molecular Imaging and Biology*. Springer, 14(5), pp. 523–533.

Leitinger, N. and Schulman, I. G. (2013) 'Phenotypic polarization of macrophages in atherosclerosis', *Arteriosclerosis, thrombosis, and vascular biology*. Am Heart Assoc, 33(6), pp. 1120–1126.

Levi, M., van der Poll, T. and Büller, H. R. (2004) 'Bidirectional relation between inflammation and coagulation', *Circulation*. Am Heart Assoc, 109(22), pp. 2698–2704.

Li, Y., Cummins, J. and Huard, J. (2001) 'Muscle injury and repair', *Current opinion in orthopaedics*. LWW, 12(5), pp. 409–415.

Li, Y., Lee, P. Y. and Reeves, W. H. (2010) 'Monocyte and macrophage abnormalities in systemic lupus erythematosus', *Archivum immunologiae et therapiae experimentalis*. Springer, 58(5), pp. 355–364.

Li, Z., Dong, X., Wang, Z., Liu, W., Deng, N., Ding, Y., Tang, L., Hla, T., Zeng, R. and Li, L. (2005) 'Regulation of PTEN by Rho small GTPases', *Nature cell biology*. Nature Publishing Group, 7(4), pp. 399–404.

Mantovani, A., Sica, A., Sozzani, S., Allavena, P., Vecchi, A. and Locati, M. (2004) 'The chemokine system in diverse forms of macrophage activation and polarization', *Trends in immunology*. Elsevier, 25(12), pp. 677–686.

Massimino, M. L., Rapizzi, E., Cantini, M., Dalla Libera, L., Mazzoleni, F., Arslan, P. and Carraro, U. (1997) 'ED2+ macrophages increase selectively myoblast proliferation in muscle cultures', *Biochemical and biophysical research communications*. Elsevier, 235(3), pp. 754–759.

Menke, J., Rabacal, W. A., Byrne, K. T., Iwata, Y., Schwartz, M. M., Stanley, E. R., Schwarting, A. and Kelley, V. R. (2009) 'Circulating CSF-1 promotes monocyte and macrophage phenotypes that enhance lupus nephritis', *Journal of the American Society of Nephrology*. Am Soc Nephrol, 20(12), pp. 2581–2592.

Mia, S., Warnecke, A., Zhang, X., Malmström, V. and Harris, R. A. (2014) 'An optimized Protocol for Human M2 Macrophages using M-CSF and IL-4/IL-10/TGF-β Yields a Dominant Immunosuppressive Phenotype', *Scandinavian journal of immunology*. Wiley Online Library, 79(5), pp. 305–314.

Mills, C. (2012) 'M1 and M2 macrophages: oracles of health and disease', *Critical Reviews™ in Immunology*. Begel House Inc., 32(6).

Mondal, S., Subramanian, K. K., Sakai, J., Bajrami, B. and Luo, H. R. (2012) 'Phosphoinositide lipid phosphatase SHIP1 and PTEN coordinate to regulate cell migration and adhesion', *Molecular biology of the cell*. Am Soc Cell Biol, 23(7), pp. 1219–1230.

Morillas-Ruiz, J. M., García, J. A. V., López, F. J., Vidal-Guevara, M. L. and Zafrilla, P. (2006) 'Effects of polyphenolic antioxidants on exercise-induced oxidative stress', *Clinical Nutrition*. Elsevier, 25(3), pp. 444–453.

Myburgh, K. H., Kruger, M. J. and Smith, C. (2012) 'Accelerated skeletal muscle recovery after in vivo polyphenol administration', *The Journal of nutritional biochemistry*. Elsevier, 23(9), pp. 1072–1079.

Naghavi, M., Wang, H., Lozano, R., Davis, A., Liang, X., Zhou, M., Vollset, S. E., Ozgoren, A. A., Abdalla, S. and Abd-Allah, F. (2015) 'Global, regional, and national age-sex specific all-cause and cause-specific mortality for 240 causes of death, 1990-2013: a systematic analysis for the Global Burden of Disease Study 2013', *Lancet*. Elsevier Science Inc, 385(9963), pp. 117–171.

Nishio, M., Watanabe, K., Sasaki, J., Taya, C., Takasuga, S., Iizuka, R., Balla, T., Yamazaki, M., Watanabe, H. and Itoh, R. (2007) 'Control of cell polarity and motility by the PtdIns (3, 4, 5) P3 phosphatase SHIP1', *Nature Cell Biology*. Nature Publishing Group, 9(1), pp. 36–44.

Noh, J. S., Kim, H. J., Kwon, M. J. and Song, Y. O. (2009) 'Active principle of kimchi, 3-(4′-hydroxyl-3′, 5′-dimethoxyphenyl) propionic acid, retards fatty streak formation at aortic sinus of apolipoprotein E knockout mice', *Journal of medicinal food*. Mary Ann Liebert, Inc. 140 Huguenot Street, 3rd Floor New Rochelle, NY 10801 USA, 12(6), pp. 1206–1212.

Noratto, G. D., Angel-Morales, G., Talcott, S. T. and Mertens-Talcott, S. U. (2011) 'Polyphenolics from açaí (Euterpe oleracea Mart.) and red muscadine grape (Vitis rotundifolia) protect human umbilical vascular Endothelial cells (HUVEC) from glucose-and lipopolysaccharide (LPS)-induced inflammation and target microRNA-126', *Journal of agricultural and food chemistry*. ACS Publications, 59(14), pp. 7999–8012.

Papakonstanti, E. A., Ridley, A. J. and Vanhaesebroeck, B. (2007) 'The p110δ isoform of PI 3-kinase negatively controls RhoA and PTEN', *The EMBO journal*. EMBO Press, 26(13), pp. 3050–3061.

Parsa, R., Andresen, P., Gillett, A., Mia, S., Zhang, X.-M., Mayans, S., Holmberg, D. and Harris, R. A. (2012) 'Adoptive transfer of immunomodulatory M2 macrophages prevents type 1 diabetes in NOD mice', *Diabetes*. Am Diabetes Assoc, 61(11), pp. 2881–2892.

Pestonjamasp, K. N., Forster, C., Sun, C., Gardiner, E. M., Bohl, B., Weiner, O., Bokoch, G. M. and Glogauer, M. (2006) 'Rac1 links leading edge and uropod events through Rho and myosin activation during chemotaxis', *Blood*. Am Soc Hematology, 108(8), pp. 2814–2820.

Petersen, K. S. and Smith, C. (2016) 'Ageing-associated oxidative stress and inflammation are alleviated by products from grapes', *Oxidative Medicine and Cellular Longevity*, 2016. doi: 10.1155/2016/6236309.

Petridou, A., Chatzinikolaou, A., Fatouros, I., Mastorakos, G., Mitrakou, A., Chandrinou, H., Papassotiriou, I. and Mougios, V. (2007) 'Resistance exercise does not affect the serum concentrations of cell adhesion molecules', *British journal of sports medicine*. BMJ Publishing Group Ltd and British Association of Sport and Exercise Medicine, 41(2), pp. 76–79.

Petrie, R. J., Doyle, A. D. and Yamada, K. M. (2009) 'Random versus directionally persistent cell migration', *Nature reviews Molecular cell biology*. Nature Publishing Group, 10(8), pp. 538–549.

Roberts, C. K., Won, D., Pruthi, S., Lin, S. S. and Barnard, R. J. (2006) 'Effect of a diet and exercise intervention on oxidative stress, inflammation and monocyte adhesion in diabetic

men', *Diabetes Research and Clinical Practice*, 73(3), pp. 249–259. doi: 10.1016/j.diabres.2006.02.013.

Ruf, W., Dorfleutner, A. and Riewald, M. (2003) 'Specificity of coagulation factor signaling', *Journal of Thrombosis and Haemostasis*. Wiley Online Library, 1(7), pp. 1495–1503.

Saclier, M., Yacoub-Youssef, H., Mackey, A. L., Arnold, L., Ardjoune, H., Magnan, M., Sailhan, F., Chelly, J., Pavlath, G. K., Mounier, R., Kjaer, M. and Chazaud, B. (2013) 'Differentially activated macrophages orchestrate myogenic precursor cell fate during human skeletal muscle regeneration', *Stem Cells*, 31(2), pp. 384–396. doi: 10.1002/stem.1288.

Sankhari, J. M., Thounaojam, M. C., Jadeja, R. N., Devkar, R. V and Ramachandran, A. V (2012) 'Anthocyanin-rich red cabbage (Brassica oleracea L.) extract attenuates cardiac and hepatic oxidative stress in rats fed an atherogenic diet', *Journal of the Science of Food and Agriculture*. Wiley Online Library, 92(8), pp. 1688–1693.

Sapey, E., Greenwood, H., Walton, G., Mann, E., Love, A., Aaronson, N., Insall, R. H., Stockley, R. A. and Lord, J. M. (2014) 'Phosphoinositide 3-kinase inhibition restores neutrophil accuracy in the elderly: toward targeted treatments for immunosenescence', *Blood*. Am Soc Hematology, 123(2), pp. 239–248.

Sapey, E., Stockley, J. A., Greenwood, H., Ahmad, A., Bayley, D., Lord, J. M., Insall, R. H. and Stockley, R. A. (2011) 'Behavioral and structural differences in migrating peripheral neutrophils from patients with chronic obstructive pulmonary disease', *American journal of respiratory and critical care medicine*. Am Thoracic Soc, 183(9), pp. 1176–1186.

Schabbauer, G., Matt, U., Günzl, P., Warszawska, J., Furtner, T., Hainzl, E., Elbau, I., Mesteri, I., Doninger, B. and Binder, B. R. (2010) 'Myeloid PTEN promotes inflammation but impairs bactericidal activities during murine pneumococcal pneumonia', *The Journal of Immunology*. Am Assoc Immnol, 185(1), pp. 468–476.

Schleimer, R. P., Sterbinsky, S. A., Kaiser, J., Bickel, C. A., Klunk, D. A., Tomioka, K., Newman, W., Luscinskas, F. W., Gimbrone, M. A. and McIntyre, B. W. (1992) 'IL-4 induces adherence of human eosinophils and basophils but not neutrophils to endothelium. Association with expression of VCAM-1.', *The Journal of Immunology*. Am Assoc Immnol, 148(4), pp. 1086–1092.

Schmidt, T., Zündorf, J., Grüger, T., Brandenburg, K., Reiners, A.-L., Zinserling, J. and

Schnitzler, N. (2012) 'CD66b overexpression and homotypic aggregation of human peripheral blood neutrophils after activation by a gram-positive stimulus', *Journal of leukocyte biology*. Soc Leukocyte Biology, 91(5), pp. 791–802.

Sen, C. K. and Bagchi, D. (2001) 'Regulation of inducible adhesion molecule expression in human endothelial cells by grape seed proanthocyanidin extract', *Molecular and cellular biochemistry*. Springer, 216(1–2), pp. 1–7.

Servant, G., Weiner, O. D., Herzmark, P., Balla, T., Sedat, J. W. and Bourne, H. R. (2000) 'Polarization of chemoattractant receptor signaling during neutrophil chemotaxis', *Science*. American Association for the Advancement of Science, 287(5455), pp. 1037–1040.

Sherwood, E. R. and Toliver-Kinsky, T. (2004) 'Mechanisms of the inflammatory response', *Best Practice & Research Clinical Anaesthesiology*. Elsevier, 18(3), pp. 385–405.

Sherwood, L. (2015) *Human physiology: from cells to systems*. Cengage learning.

Shi, Y., Zhang, J., Mullin, M., Dong, B., Alberts, A. S. and Siminovitch, K. A. (2009) 'The mDial formin is required for neutrophil polarization, migration, and activation of the LARG/RhoA/ROCK signaling axis during chemotaxis', *The Journal of Immunology*. Am Assoc Immnol, 182(6), pp. 3837–3845.

Sica, A. and Mantovani, A. (2012) 'Macrophage plasticity and polarization: in vivo veritas', *The Journal of clinical investigation*. Am Soc Clin Investig, 122(3), pp. 787–795.

Smith, C., Kruger, M. J., Smith, R. M. and Myburgh, K. H. (2008) 'The inflammatory response to skeletal muscle injury', *Sports medicine*. Springer, 38(11), pp. 947–969.

Sonnet, C., Lafuste, P., Arnold, L., Brigitte, M., Poron, F., Authier, F., Chrétien, F., Gherardi, R. K. and Chazaud, B. (2006) 'Human macrophages rescue myoblasts and myotubes from apoptosis through a set of adhesion molecular systems', *Journal of cell science*. The Company of Biologists Ltd, 119(12), pp. 2497–2507.

Srinivasan, S., Wang, F., Glavas, S., Ott, A., Hofmann, F., Aktories, K., Kalman, D. and Bourne, H. R. (2003) 'Rac and Cdc42 play distinct roles in regulating PI (3, 4, 5) P3 and polarity during neutrophil chemotaxis', *The Journal of cell biology*. Rockefeller Univ Press, 160(3), pp. 375–385.

Stegeman, C. A., Tervaert, J. W. C., Huitema, M. G., Jong, P. E. De and Kallenberg, C. G. M. (1994) 'Serum levels of soluble adhesion molecules intercellular adhesion molecule 1,

vascular cell adhesion molecule 1, and E-selectin in patients with wegener's granulomatosis. Relationship to disease activity and relevance during followup', *Arthritis & Rheumatism*. Wiley Online Library, 37(8), pp. 1228–1235.

Strassheim, D., Kim, J.-Y., Park, J.-S., Mitra, S. and Abraham, E. (2005) 'Involvement of SHIP in TLR2-induced neutrophil activation and acute lung injury', *The Journal of Immunology*. Am Assoc Immnol, 174(12), pp. 8064–8071.

Subramanian, K. K., Jia, Y., Zhu, D., Simms, B. T., Jo, H., Hattori, H., You, J., Mizgerd, J. P. and Luo, H. R. (2007) 'Tumor suppressor PTEN is a physiologic suppressor of chemoattractant-mediated neutrophil functions', *Blood*. Am Soc Hematology, 109(9), pp. 4028–4037.

Summan, M., Warren, G. L., Mercer, R. R., Chapman, R., Hulderman, T., Van Rooijen, N. and Simeonova, P. P. (2006) 'Macrophages and skeletal muscle regeneration: a clodronate-containing liposome depletion study', *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*. Am Physiological Soc, 290(6), pp. R1488–R1495.

Tan, H.-Y., Wang, N., Li, S., Hong, M., Wang, X. and Feng, Y. (2016) 'The Reactive Oxygen Species in Macrophage Polarization: Reflecting Its Dual Role in Progression and Treatment of Human Diseases', *Oxidative Medicine and Cellular Longevity*, 2016, pp. 1–16. doi: 10.1155/2016/2795090.

Thornhill, M. H. and Haskard, D. O. (1990) 'IL-4 regulates endothelial cell activation by IL-1, tumor necrosis factor, or IFN-gamma.', *The Journal of Immunology*. Am Assoc Immnol, 145(3), pp. 865–872.

Tidball, J. G. (2005) 'Inflammatory processes in muscle injury and repair', *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*. Am Physiological Soc, 288(2), pp. R345–R353.

Toumi, H. and Best, T. M. (2003) 'The inflammatory response: friend or enemy for muscle injury?', *British journal of sports medicine*. BMJ Publishing Group Ltd and British Association of Sport and Exercise Medicine, 37(4), pp. 284–286.

Vanhaesebroeck, B., Leevers, S. J., Ahmadi, K., Timms, J., Katso, R., Driscoll, P. C., Woscholski, R., Parker, P. J. and Waterfield, M. D. (2001) 'Synthesis and function of 3-phosphorylated inositol lipids', *Annual review of biochemistry*. Annual Reviews 4139 El Camino Way, PO Box 10139, Palo Alto, CA 94303-0139, USA, 70(1), pp. 535–602.

Velasco, P., Francisco, M., Moreno, D. A., Ferreres, F., García-Viguera, C. and Cartea, M. E. (2011) 'Phytochemical fingerprinting of vegetable Brassica oleracea and Brassica napus by simultaneous identification of glucosinolates and phenolics', *Phytochemical Analysis*. Wiley Online Library, 22(2), pp. 144–152.

Vemula, S., Shi, J., Hanneman, P., Wei, L. and Kapur, R. (2010a) 'ROCK1 functions as a suppressor of inflammatory cell migration by regulating PTEN phosphorylation and stability', *Blood*, 115(9), pp. 1785–1796. doi: 10.1182/blood-2009-08-237222.

Vemula, S., Shi, J., Hanneman, P., Wei, L. and Kapur, R. (2010b) 'ROCK1 functions as a suppressor of inflammatory cell migration by regulating PTEN phosphorylation and stability', *Blood*. Am Soc Hematology, 115(9), pp. 1785–1796.

Wållberg, M. and Harris, R. A. (2005) 'Co-infection with Trypanosoma brucei brucei prevents experimental autoimmune encephalomyelitis in DBA/1 mice through induction of suppressor APCs', *International immunology*. Jpn Soc Immunol, 17(6), pp. 721–728.

Wang, F. (2009) 'The signaling mechanisms underlying cell polarity and chemotaxis', *Cold Spring Harbor perspectives in biology*. Cold Spring Harbor Lab, 1(4), p. a002980.

Weiner, O. D., Neilsen, P. O., Prestwich, G. D., Kirschner, M. W., Cantley, L. C. and Bourne, H. R. (2002) 'A PtdInsP3-and Rho GTPase-mediated positive feedback loop regulates neutrophil polarity', *Nature cell biology*. Nature Publishing Group, 4(7), pp. 509–513.

Weiner, O. D., Servant, G., Welch, M. D., Mitchison, T. J., Sedat, J. W. and Bourne, H. R. (1999) 'Spatial control of actin polymerization during neutrophil chemotaxis', *Nature cell biology*. Nature Publishing Group, 1(2), pp. 75–81.

Worthylake, R. A., Lemoine, S., Watson, J. M. and Burridge, K. (2001) 'RhoA is required for monocyte tail retraction during transendothelial migration', *The Journal of cell biology*. Rockefeller Univ Press, 154(1), pp. 147–160.

Xu, J., Wang, F., Van Keymeulen, A., Herzmark, P., Straight, A., Kelly, K., Takuwa, Y., Sugimoto, N., Mitchison, T. and Bourne, H. R. (2003) 'Divergent signals and cytoskeletal assemblies regulate self-organizing polarity in neutrophils', *Cell*. Elsevier, 114(2), pp. 201–214.

Xu, W., Wang, P., Petri, B., Zhang, Y., Tang, W., Sun, L., Kress, H., Manes, T. D., Shi, Y. and Kubes, P. (2010) 'Integrin-induced PIP5K1C kinase polarization regulates neutrophil

polarization, directionality, and in vivo infiltration', *Immunity*. Elsevier, 33(3), pp. 340–350.

Yoneda, A., Multhaupt, H. A. B. and Couchman, J. R. (2005) 'The Rho kinases I and II regulate different aspects of myosin II activity', *The Journal of cell biology*. Rockefeller Univ Press, 170(3), pp. 443–453.

Zandi, S., Nakao, S., Chun, K.-H., Fiorina, P., Sun, D., Arita, R., Zhao, M., Kim, E., Schueller, O. and Campbell, S. (2015) 'ROCK-isoform-specific polarization of macrophages associated with age-related macular degeneration', *Cell reports*. Elsevier, 10(7), pp. 1173–1186.

Zhou, L., Somasundaram, R., Nederhof, R. F., Dijkstra, G., Faber, K. N., Peppelenbosch, M. P. and Fuhler, G. M. (2012) 'Impact of human granulocyte and monocyte isolation procedures on functional studies', *Clinical and Vaccine Immunology*. Am Soc Microbiol, 19(7), pp. 1065–1074.

7. ADDENDUMS

Addendum A: SOP FOR PHLEBOTOMY

- Drawing blood
- Make sure the working area is quiet, clean and well-lit
- Assemble the supplies (Blood collection tubes that have not expired, a tourniquet / band, cotton balls, adhesive tape and alcohol wipes)
- Select the appropriate needle (10ml needle)
- Bring patient into the exercise physiology lab and allow them to have a seat on the bed.
- You or the patient can decide which arm to draw blood from.
- Tie the tourniquet around the patient's arm about 7.5-10cm from the venepuncture site
- The patient needs to form a tight fist to expose the vein at the antecubital area
- Trace patients veins with index finger and tap the vein to encourage dilation
- The antecubital area is wiped clean with a alcohol wipe
- Allow 30sec for disinfected are to dry and avoid the sting of the needle.
- Thread the needle into the holder
- Tap any tubes the contain additives to dislodge the additives from the walls of the tubes
- Insert the blood collection tubes into the holder.
- Grasp your patient's arm, your thumb should pull skin 2.5-5cm below the puncture site
- Line up the needle with the vein

- Insert needle into the vein, push the collection tube toward the holder until the butt end of the needle pierces the stopper on the tube. Tube needs to be below puncture site.
- Allow tube to fill, and remove tourniquet as soon as blood flow into tube is adequate.
- Remove tube from holder and mix the contents if tube contains additives, by inverting the tube 5-8 times. Do not shake vigorously.
- Fill the remaining tubes.
- Ask patient to open his or her hand, place cotton balls over puncture site and close with adhesive tape. Apply pressure.
- 4 x 5ml sample is drawn from antecubital area of the arm with a sterile 10ml needle.
    - 2 x 5ml blood anti-coagulated in sodium heparin tube (neutrophil migration assay and IHC)
    - 2 x 5ml EDTA (for macrophage isolation)

Addendum B: SOP FOR MONOCYTE ISOLATION

Isolation of Human Monocytes by Double Gradient Centrifugation (Article from JoVE)

To facilitate balancing of the centrifuge, it is recommended to process two whole blood samples in parallel. However, take care to use separate materials for each donor and not to mix the cells. Round about $7.5x10^5$ monocytes are obtained from 4 ml of EDTA peripheral blood.

Isolation from donor whole blood

- Carefully disinfect the EDTA vacutainers containing the peripheral whole blood and transfer the contents to sterile plastic tubes. Do not mix blood from different donors.
- For 2 EDTA vacutainers (8 ml blood) fill two 15 ml tubes with 3 ml Ficoll/Histopaque solution (1.077 g/ml). The Ficoll should be at room temperature for the preparation.
- For 4 EDTA vacutainers (16 ml blood) fill two 15 ml tubes with 6 ml Ficoll/Histopaque etc.
- Layer 3-6 ml whole blood on top of the Ficoll solution for the first density gradient. Be careful to do this slowly and carefully in order to prevent mixing both layers.
- Note: Maintain Ficoll:blood ratio at 1:1-2 and Defrost FBS
- Centrifuge at 400 x g without brake for 30 min at room temperature.
- For each gradient collect the white ring of peripheral blood mononuclear cells (PBMCs) which is located between the two phases with a plastic Pasteur pipette and transfer to a 15 ml tube.
- Fill each tube with 1 mM PBS-EDTA (1x PBS and 0.5 M EDTA) up to 12 ml in total.
- Centrifuge at 300 x g for 10 min without brake at room temperature.
- Aspirate supernatant and wash pellet again with 12 ml PBS-EDTA.
- For each donor pool the pellets in 6 ml RPMI-1640 without phenol red + 10% FBS.

- Prepare the iso-osmotic Percoll solution for the second density gradient: For two donors mix 6.939 ml Percoll solution (density: 1.131 g/ml) in a 15 ml tube with 0.561

ml 10x PBS. Then transfer 6.9 ml of this solution to a new 15 ml tube and add 8.1 ml RPMI-1640 with phenol red + 10% FBS to obtain a 46% iso-osmotic Percoll solution. The Percoll should be at room temperature for the preparation.

- For each donor transfer 7ml of the prepared Percoll solution to a 15 ml tube and layer the PBMC solution prepared in step 11. on top of the Percoll solution. Be careful to do this very slowly and carefully, both layers tend to mix easily. If done correctly the two phases can be distinguished due to their difference in colour.
- Centrifuge at 550 x g without brake for 30 min at room temperature.
- For each gradient collect the white ring of monocytes which is located between the two phases with a plastic Pasteur pipette and transfer to a 15 ml tube.
- Fill each tube with PBS-EDTA up to the 14 ml mark.
- Centrifuge at 400 x g for 10 min without brake at room temperature.
- Aspirate the supernatant and resuspend in 1ml cytofix/cytoperm
- Leave in fridge at 4°C for 20min
- Centrifuge 4°C, 5min at 400xg
- Remove supernatant and resuspend in 1ml cytoperm/wash. X2
- Remove supernatant and resuspend in 1ml plain PBS
- Aliquot 480ul into two different eppi's (unstained and multiple stained) add the difference in PBS to make up 1ml
- Switch lights off in the hood. Add all the antibodies, 5ul CD206, 5ul CD274, 5ul CD153, 20ul CD86, 5ul IL-10, 5ul HD-LA, 20ul MPO to multiple stained eppi and 65ul PBS in unstained eppi.
- Close with tinfoil and place in incubator for 10min
- Centrifuge 1600rpm for 5min
- Resuspend in 400ul PBS and store in fridge at 4°C overnight

Addendum C: SOP FOR NEUTROPHIL ISOLATION FOR FLOW CYTOMETRY

- 2ml Histopaque1077(room temperature) is pipette into a 15ml falcon tube and then double volume (4ml) of anticoagulant added blood was carefully poured on this Histopaque layer down the side of each tube.
- Tubes are transferred to the centrifuge machine with swinging rotar and are spun at speed of 652xg for 30 minutes at 21°C, 1800rpm.

* Four different layers of blood are formed*

- Sediment containing neutrophils and erythrocytes is taken by discarding the supernatant layer containing lymphocytes, monocytes and plasma layer on top respectively.
- Sediment is mixed with equal volume of 6% (room temperature) dextran (in Normal Saline) and incubated at 37°C for 45 minutes to allow the sedimentation of erythrocytes.
- Pellet containing erythrocytes is discarded and the neutrophils rich supernatant is collected and mixed with 10ml of PBS.
- Reaction mixture is centrifuged at 290xg for 10 minutes at 4°C, 1500rpm.
- The pellet containing the neutrophils was taken by removing the supernatant.
- Washing with 1ml PBS, repeat once or twice depending on the amount of erythrocytes present as a contaminant.
- The supernatant was removed by taking the pellet.
- Resuspend pellet in 1.2ml RPMI, Aliquot 200ul into eppi to perform a cell count. (Use the cell dyne)
- The final neutrophils pellet was resuspended into desired volume of media (RMPI) to achieve the appropriate cell concentration usually $1x10^{-6}$ cells/ ml.
- Spin down for 10 minutes at 4°C, 1500rpm.
- Remove supernatant and resuspend pellet in PBS (eg. If the cell down gave a count of $2.7x10^{-6}$ cells/ ml and we want $1x10^{-6}$ cells/ ml the resuspend pellet in 270ul of PBS).

- Defrost Paraformaldehyde.
- Depending on the amount of stains you need to do, try aliquot 100ul with $1x10^{-6}$ cells/ ml into two eppi's (multiple stained and the unstained)
- Switch light off in the hood once you start staining. Add 20ul surface marker antibody CD66b to the multiple stained eppi and 20ul PBS in the unstained eppi.
- Vortex gently
- Leave in fridge for 30min at 4°C covered in tinfoil
- Add 500ul PBS
- Vortex gently
- Centrifuge at 1800rmp for 6min
- Remove supernatant and resuspend in 250ul cytofix/cytoperm
- Mix gently
- Leave in fridge for 25min at 4°C covered in tinfoil.
- Add 500ul BD perm wash
- Vortex gently
- Centrifuge at 1800rpm for 6min
- Remove supernatant
- Add intracellular markers 5ul ICAM and 5ul VCAM to the multiple stain, add 10ul PBS to the unstained.
- Leave in fridge for 30min at 4°C covered in tinfoil
- Repeat wash with 250ul BD perm wash
- Vortex gently
- Centrifuge at 1800rpm for 6min
- Remove supernatant

- Resuspend in 500ul 4% paraformaldehyde.
- Leave in fridge at 4°C overnight, covered in foil
- Perform flow cytometry analysis in the morning

Addendum D: SOP NEUTROPHIL ISOLATION FOR 8 CHAMBER SLIDE/WELL

- 2ml Histopaque1077(room temperature) is pipette into a 15ml falcon tube and then double volume (4ml) of anticoagulant added blood was carefully poured on this Histopaque layer down the side of each tube.
- Tubes are transferred to the centrifuge machine with swinging rotar and are spun at speed of 652xg for 30 minutes at 21°C, 1800rpm.

* Four different layers of blood are formed*

- Sediment containing neutrophils and erythrocytes is taken by discarding the supernatant layer containing lymphocytes, monocytes and plasma layer on top respectively.
- Sediment is mixed with equal volume of 6% (room temperature) dextran (in Normal Saline) and incubated at 37°C for 45 minutes to allow the sedimentation of erythrocytes.
- 8 well plate needs to be activated, 30min open under a UV light.
- Pellet containing erythrocytes is discarded and the neutrophils rich supernatant is collected and mixed with 10ml of PBS.
- Reaction mixture is centrifuged at 290xg for 10 minutes at 4°C, 1500rpm.
- The pellet containing the neutrophils was taken by removing the supernatant.
- Washing with 1ml PBS, repeat once or twice depending on the amount of erythrocytes present as a contaminant.
- The supernatant was removed by taking the pellet.
- Resuspend pellet in 1.2ml RPMI, Aliquot 200ul into eppi to perform a cell count. (Use the cell dyne)
- The final neutrophils pellet was resuspended into desired volume of media (RMPI) to achieve the appropriate cell concentration usually $1x10^{-6}$ cells/ ml.
- Add 75ul to the bottom left hand corner of each well in the 8 well plate.

- Take 8 well plate to the Olympus microscope and mount the 8 well plate.

- Add Fmlp to the opposite (top right hand corner) this will allow for chemotaxis to take place while a video can be made tracking the neutrophils movements. (If comparing a chemoattractant to a control, replace the Fmlp with RPMI).
- Track neutrophils for 45min on Olympus microscope.

Addendum E: SOP FOR IMMUNOHISTOCHEMISTRY

- Fix cells in 4% paraformaldehyde (75ul and 75ul RPMI) in each well, incubate for 10min at 37°C.
- Wash with 100ul PBS x3 per well
- Permeabilized with 0.1% Triton X-100 for 5min
- Wash with 200ul PBS, leave for 5min
- Block with 150ul 1% BSA, leave for 20min
- Add Primary conjugated antibody (100ul in total). Leave in fridge at 4°C covered in tinfoil overnight.
- Anti-ROCK (488) dilution 1:50 (10ul in 500ul PBS)
- Anti-PI3K (647) dilution 1:70 (7.1ul in 500ul PBS)
- Wash with cold PBS x 3
- Add 100ul DAPPI for 10min
- Wash with cold PBS x3
- Leave 100ul PBS in each well for Visualization
- Visualize with confocal microscope.

Addendum F: PARTICIPANT INFORMATION LEAFLET

TITLE OF THE RESEARCH PROJECT:

REFERENCE NUMBER:

PRINCIPAL INVESTIGATOR: Tanya Smith

ADDRESS: Dept Physiological Sciences, Stellenbosch University, Private Bag X1, Matieland, 7602 OR Dept Physiological Sciences, Room 2014, Mike de Vries building, Merriman Avenue, Main Campus, Stellenbosch

CONTACT NUMBER: 0733766440

Dear Colleague *(fellow student, etc)*

My name is Tanya Smith and I am a master's student and I would like to invite you to participate in a research project that aims to investigate changes in experimentally induced chemokinetic responses after *in vivo* supplementation with grape seed-derived proanthocyanidolic oligomers (PCO).

Please take some time to read the information presented here, which will explain the details of this project and contact me if you require further explanation or clarification of any aspect of the study. Also, your participation is entirely voluntary and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part.

This study has been approved by the Health Research Ethics Committee (HREC) at Stellenbosch University and will be conducted according to accepted and applicable National and International ethical guidelines and principles, including those of the international Declaration of Helsinki October 2008.

Description of the study:

Since the study is designed to be largely in vitro, all participants will be required to follow the subject requirements. We will obtain a resting blood sample (5ml blood anti-coagulated in sodium heparin and 5ml EDTA, as well as 5ml sodium citrate) from individuals prior to the two week study. Baseline parameters for each individual will be determined. The participants will then be placed on either the placebo or PCO supplement for two weeks. A second blood sample will be taken after 7 days to monitor full blood count and clotting profile only. The last blood sample will be taking at the end of the two week study. All blood will be drawn by an experienced phlebotomist from a forearm vein.

Clotting profile: A full blood count will be done.

We will be looking at neutrophil migration patterns using live cell tracking and immunofluorescence staining. As well as the role of adhesion molecules and the impact of macrophages via low cytometry.

Study population

We will recruit both male and female individuals between 18-25 years of age. For recruitment, we will invite volunteers by advertisements on notice boards on campus. Refer to appendix A for an example of such an advertisement. Ideally, a total of 20 individuals will complete the protocol.

Inclusion criteria would be any person that are normally healthy, not on any other type of supplements, recreationally active and non-smoking. Exclusion criteria: Individuals with known inflammatory disease, or chronic disease or known to have any sort of inflammatory component such as sinus or hayfever suffers, will be excluded from the study. Subjects will be required to refrain from taking vitamin or anti-oxidant supplements for a period of 14 days prior to initiation of the protocol, as well as for the duration of the entire protocol. All subjects will be required to refrain from exercise for a period of 24 hours prior to each sample collection time point

Anticipated benefits, risks and precautions

PCO could prove to be a cost-effective way of modulating inflammation beneficially. Participants will be informed at the end of the study whether they received the real

supplement or placebo and will be given the option to take a free 2-week supply of the product, if we have shown it to have beneficial effects as anticipated.

The normal, minimal risk of bruising and infection associated with blood draws will exist, but since experienced phlebotomists will be taking blood samples, we are confident that we have minimised this risk. In addition, since blood clotting has adhesion molecule involvement, there is theoretically a small risk of mild hypo-coagulability. Although no anecdotal reports of bleeding episodes have been received from users of this product or participants in previous studies by our group, we will monitor clotting profiles weekly.

Ethical considerations

We are confident that we have optimised subject number to ensure sufficient statistical power, while limiting subject number. In order to maintain confidentiality, all samples will be labelled using code known only to the principal and sub-investigator.

We have the necessary human resources, infrastructure and expertise in place to perform this study. We are confident that the anticipated benefits from this study outweighs the minor risks by far.

If you are willing to participate in this study please sign the attached Declaration of Consent and *(hand it to the investigator)*

Yours sincerely

Tanya Smith

Principal Investigator

Declaration by participant

By signing below, I ……..……. agree to take part in a research study entitled ……..

.

I declare that:

- I have read the attached information leaflet and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is voluntary and I have not been pressurised to take part.
- I may choose to leave the study at any time and will not be penalised or prejudiced in any way.
- I may be asked to leave the study before it has finished, if the researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

Signed at (*place*) .....…..…….. On (*date*) ……...…….. 2015.

Signature of participant

Addendum G: PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM

TITLE OF THE RESEARCH PROJECT: Assessment of *in vitro* inflammatory responses after *in vivo* supplementation with grape seed-derived proanthocyanidolic oligomers (PCO).

REFERENCE NUMBER:

PRINCIPAL INVESTIGATOR: Tanya Smith

ADDRESS: Dept Physiological Sciences, Stellenbosch University, Private Bag X1, Matieland, 7602 OR Dept Physiological Sciences, Room 2014, Mike de Vries building, Merriman Avenue, Main Campus, Stellenbosch

CONTACT NUMBER: 0733766440

You are being invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how you could be involved. Also, your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part.

This study has been approved by the **Health Research Ethics Committee at Stellenbosch University** and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

What is this research study all about?

The study will be of longitudinal, placebo-controlled design and will be performed in the tissue culture and human physiology laboratories of the Department Physiological Sciences at Stellenbosch University. We will recruit both male and female individuals between 18-25 years of age. Ideally, a total of 20 individuals will complete the protocol. Given these promising effects we have been able to illustrate in the context of PCO treatment during previous studies done by our group, we would like to investigate potential mechanisms by which these anti-inflammatory effects are achieved. We propose to assess the potential of grape seed-derived PCO in this context.
Since the modulation of inflammation is not only relevant to individuals in the sporting arena or aged individuals, but also to the aetiology and thus treatment of various chronic disease states, this study is of high relevance to our society as a whole. Inclusion criteria would be any person that are normally healthy, not on any other type of supplements, recreationally active and non-smoking.

Sample collection: Since the study is designed to be largely in vitro, all participants will be required to follow the subject requirements. We will obtain a resting blood sample (5ml blood anti-coagulated in sodium heparin and 5ml EDTA, as well as 5ml sodium citrate) from individuals prior to the two week study. Baseline parameters for each individual will be determined. The participants will then be placed on either the placebo or PCO supplement for two weeks. A second blood sample will be taken after 7 days to monitor full blood count and clotting profile only. The last blood sample will be taken at the end of the supplementation period. All blood will be drawn by an experienced phlebotomist from a forearm vein.

Sample analysis

Full blood count: A full blood count will be performed on EDTA blood in the dept Physiological Sciences using an automated cell counter (Celldyne 3700 CS) and general haematology and immunology profile will be assessed by experienced medical technologist registered with the HPSCA (Prof C Smith).

The citrate blood will be analysed for clotting profile by PathCare Stellenbosch, using standard laboratory practise.

Why have you been invited to participate?

You are between the age 18-25, you are not on any supplements, you do not smoke and you do not suffer from inflammatory disease or chronic disease linked to an inflammatory component such as sinus/ hayfever suffers.

What will your responsibilities be?

Subjects will be required to refrain from taking vitamin or anti-oxidant supplements as well as any strenuous exercise for a period of 14 days prior to initiation of the protocol, as well as for the duration of the entire protocol. All subjects will be required to refrain from exercise for a period of 24 hours prior to each sample collection time point.

Will you benefit from taking part in this research?

PCO could prove to be a cost-effective way of modulating inflammation beneficially. Participants will be informed at the end of the study whether they received the real supplement or placebo and will be given the option to take a free 2-week supply of the product, if we have shown it to have beneficial effects as anticipated. However participants might not experience the full benefit as the duration period might be a factor, but future participants will.

Are there in risks involved in your taking part in this research?

The normal, minimal risk of bruising and infection associated with blood draws will exist, but since experienced phlebotomists will be taking blood samples, we are confident that we have minimised this risk. In addition, since blood clotting has adhesion molecule involvement, there is theoretically a small risk of mild hypocoagulability in PCO-supplemented individuals. Although no anecdotal reports of bleeding episodes have been received from users of this product or participants in previous studies by our group, we will monitor clotting

profiles weekly. In the unlikely event of a participant exhibiting abnormal clotting times, supplementation will be discontinued straight away.

If you do not agree to take part, what alternatives do you have?

There is no alternative protocol.

Who will have access to your medical records?

In order to maintain confidentiality, all samples will be labelled using code known only to the principal and sub-investigator, but we will not have access to your medical records.

What will happen in the unlikely event of some form injury occurring as a direct result of your taking part in this research study?

In the unlikely event of some form of injury occurring a departmental investigation will be done and necessary steps shall be taken keeping into consideration the participants best interests.

Will you be paid to take part in this study and are there any costs involved?

No you will not be paid to take part in the study but your transport and meal costs will be covered for each study visit. There will be no costs involved for you, if you do take part.

Is there anything else that you should know or do?

- You can contact Dr Carine Smith at tel 021-808 3146 if you have any further queries or encounter any problems.
- You can contact the Health Research Ethics Committee at 021-938 9207 if you have any concerns or complaints that have not been adequately addressed by your study doctor.
- You will receive a copy of this information and consent form for your own records.

Declaration by participant

By signing below, I ……..……. agree to take part in a research study entitled *(insert title of study).*

I declare that:

- I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.
- I may choose to leave the study at any time and will not be penalised or prejudiced in any way.

- I may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

Signed at (*place*) .....…..…….. on (*date*) ……...…….. 2015.

....

Signature of participant **Signature of witness**

Declaration by investigator

I *(name)* ……..…… declare that:

- I explained the information in this document to ……..
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above
- I did/did not use a interpreter. (*If a interpreter is used then the interpreter must sign the declaration below.*

Signed at (*place*) .....…..…….. on (*date*) ……...…….. 2015.

....

Signature of investigator **Signature of witness**

Declaration by interpreter

I *(name)* ……..…… declare that:

- I assisted the investigator (*name*) ……. to explain the information in this document to (*name of participant*) ……..…….. using the language medium of Afrikaans/Xhosa.
- We encouraged him/her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

Signed at (*place*) .....…..……. on (*date*) ……...……..

.....	.....
Signature of interpreter	**Signature of witness**

Addendum H: FLYER FOR RECRUITMENT

ATTENTION: SCIENCE NEEDS YOUR HELP!!

- Are you between the ages of 18-25
- Are you willing to donate 3 small blood samples to further our understanding of inflammation?
- Are you not on any types of supplements?
- Are you willing to take a commercially available natural product supplement for 2 weeks?

If you answered "YES" to the above questions, you have the opportunity to be part of a health solution!

If you would like to participate in a research study, please contact Tanya Smith, 16055683@sun.ac.za or on 0733766440

Thank you – we are waiting for your call!!

Addendum I: ETHICS APPROVAL

UNIVERSITEIT·STELLENBOSCH·UNIVERSITY
jou kennisvennoot • your knowledge partner

21-Jan-2016

Smith, Tanya T

Ethics Reference #: M15/09/040

Approved with Stipulations

Response to Modifications- (New Application)

Title: Assessment of in vitro inflammatory responses after in vivo supplementation with grape seed-derived

Dear Miss Tanya Smith, proanthocyanidolic oligomers (PCO).

The Response to Modifications - *(New Application)* received on 10-Dec-2015, was reviewed by members of Health Research Ethics Committee 1 via Expedited review procedures on 08-Jan-2016.

Please note the following information about your approved research protocol:

Protocol Approval Period: 21-Jan-2016 -20-Jan-2017

The Stipulations of your ethics approval are as follows:

GCP certificates for PI still needs to be submitted

Please remember to use your protocol number (M15/09/040) on any documents or correspondence with the HREC concerning your research protocol.

Please note that the HREC has the prerogative and authority to ask further questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

After Ethical Review:

Please note a template of the progress report is obtainable on www.sun.ac.za/rds and should be submitted to the Committee before the year has expired.

The Committee will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly for an external audit.

Translation of the consent document to the language applicable to the study participants should be submitted.

Federal Wide Assurance Number: 00001372

Institutional Review Board (IRB) Number: IRB0005239

The Health Research Ethics Committee complies with the SA National Health Act No.61 2003 as it pertains to health research and the United States

Code of Federal Regulations Title 45 Part 46. This committee abides by the ethical norms and principles for research, established by the Declaration of

Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes

Provincial and City of Cape Town Approval

Please note that for research at a primary or secondary healthcare facility permission must still be obtained from the relevant authorities (Western Cape

Department of Health and/or City Health) to conduct the research as stated in the protocol. Contact persons are Ms Claudette Abrahams at Western

Cape Department of Health (healthres@pgwc.gov.za Tel: +27 21 483 9907) and Dr Helene Visser at City Health (Helene.Visser@capetown.gov.za Tel:

+27 21 400 3981). Research that will be conducted at any tertiary academic institution requires approval from the relevant hospital manager. Ethics

Approval is required BEFORE approval can be obtained from these health authorities.

We wish you the best as you conduct your research.

For standard HREC forms and documents please visit: www.sun.ac.za/rds

If you have any questions or need further assistance, please contact the HREC office at 0219389657.

Included Documents:

Description of study site

ICF English

Cover Letter

Flow Chart

20151211 Mods Req - ICF clean copy

Supervisor CV - C Smith

Protocol

Package Insert

Protocol Synopsis

GCP letter - T Smith

Flyer for recruitment

20151210 Mods Req - Response letter

MCC letter

Certificate of Insurance

Investigator CV - T Smith

Investigator Declaration - C Smith

Investigator Declaration - T Smith

Application Form

Clinical Trials Checklist

NHREC Trial Application

20151210 Mods Req - Consent Form

Sincerely,

Franklin Weber

HREC Coordinator

Health Research Ethics Committee 12004 (Department of Health).

Investigator Responsibilities

Protection of Human Research Participants

Some of the responsibilities investigators have when conducting research involving human participants are listed below:

1.Conducting the Research. You are responsible for making sure that the research is conducted according to the HREC approved research protocol. You are also responsible for the actions of all your co-investigators and research staff involved with this research.

2.Participant Enrolment. You may not recruit or enrol participants prior to the HREC approval date or after the expiration date of HREC approval. All recruitment materials for any form of media must be approved by the HREC prior to their use. If you need to recruit more participants than was noted in your HREC approval letter, you must submit an amendment requesting an increase in the number of participants.

3.Informed Consent. You are responsible for obtaining and documenting effective informed consent using **only** the HREC-approved consent documents, and for ensuring that no human participants are involved in research prior to obtaining their informed consent. Please give all participants copies of the signed informed consent documents. Keep the originals in your secured research files for at least fifteen (15) years.

4.Continuing Review. The HREC must review and approve all HREC-approved research protocols at intervals appropriate to the degree of risk but not less than once per year. There is **no grace period.** Prior to the date on which the HREC approval of the research expires, **it is your responsibility to submit the continuing review report in a timely fashion to ensure a lapse in HREC approval does not occur.** If HREC approval of your research lapses, you must stop new participant enrolment, and contact the HREC office immediately.

5.Amendments and Changes. If you wish to amend or change any aspect of your research (such as research design, interventions or procedures, number of participants, participant population, informed consent document, instruments, surveys or recruiting material), you must submit the amendment to the HREC for review using the current Amendment Form. You **may not initiate** any amendments or changes to your research without first obtaining written HREC review and approval. The **only exception** is when it is necessary to eliminate apparent immediate hazards to participants and the HREC should be immediately informed of this necessity.

6.Adverse or Unanticipated Events. Any serious adverse events, participant complaints, and all unanticipated problems that involve risks to participants or others, as well as any research-related injuries, occurring at this institution or at other performance sites must be reported to the HREC within **five (5) days** of discovery of the incident. You must also report any instances of serious or continuing problems, or non-compliance with the HRECs requirements for protecting human research participants. The only exception to this policy is that the death of a research participant must be reported inaccordance with the Stellenbosch Universtiy Health Research Ethics Committee Standard Operating Procedures

www.sun025.sun.ac.za/portal/page/portal/Health_Sciences/English/Centres%20and%20Institutions/Research_Development_Support/Ethics/Application_package All reportable events should be submitted to the HREC using the Serious Adverse Event Report Form.

7.Research Record Keeping. You must keep the following research-related records, at a minimum, in a secure location for a minimum of fifteen years: the HREC approved research protocol and all amendments; all informed consent documents; recruiting materials; continuing review reports; adverse or unanticipated events; and all correspondence from the HREC

8.Reports to the MCC and Sponsor. When you submit the required annual report to the MCC or you submit required reports to your sponsor, youmust provide a copy of that report to the HREC. You may submit the report at the time of continuing HREC review.

9.Provision of Emergency Medical Care. When a physician provides emergency medical care to a participant without prior HREC review and approval,to the extent permitted by law, such activities will not be recognised as research nor will the data obtained by any such activities should it be used insupport of research.

10.Final reports. When you have completed (no further participant enrolment, interactions, interventions or data analysis) or stopped work on yourresearch, you must submit a Final Report to the HREC.

11.On-Site Evaluations, MCC Inspections, or Audits. If you are notified that your research will be reviewed or audited by the MCC, the sponsor, anyother external agency or any internal group, you must inform the HREC immediately of the impending audit/evaluation.